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THE EFFECT OF PB, ZN, CU AND NI ON THE EMBRYONIC AND LARVAL STAGES OF S. PURPURATUS

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TITLE: The Effect of Pb, Zn, Cu and Ni on the embryonic and larval stages of the Purple Sea Urchin *S. purpuratus*

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ABSTRACT

In light of the paucity of information on the toxicity of lead (Pb), zinc (Zn), copper (Cu) and nickel (Ni) in the marine environment, the aim of this thesis was to generate data on the mechanisms of toxicity of Pb, Zn, Cu and Ni in the early life stages of a very sensitive marine organism, the purple sea urchin (*Strongylocentrotus purpuratus*). Previous studies in other systems indicated that these metals can impact ionoregulation, especially calcium homeostasis, so an initial study focused on ionoregulatory changes during the first 96h of development under control conditions in embryonic and larval stages of *S. purpuratus*. Control tests showed that patterns of Ca, K, Na and Mg had an interesting pattern of accumulation over 96 h development and that the most pronounced changes were observed during the gastrulation stage. A variety of biological endpoints were subsequently utilized to examine potential mechanisms of toxicity. Toxicity tests were performed to determine median toxicity threshold values (i.e. EC50: median effective concentration and LA50: median lethal accumulation) for Zn and Pb. Growth, unidirectional Ca uptake rates, whole body ion concentrations (Na, K, Ca, Mg), and Ca ATPase activity were also monitored every 12 h over the first 84 or 96 h of early development to investigate the mechanisms of toxicity during acute and chronic exposures to lethal and sublethal concentrations of Pb, Zn, Cu and Ni in 100% sea water.

Sea urchin embryos were very sensitive to Zn with an EC50 of 2.3 µmol/L (95% C.I. = 1.97–2.71 µmol/L) and LA50 of 4.8 (2.16-11.33) µmol/kg. Embryos displayed even higher sensitivity to Pb with an EC50 of 0.36 (0.25-0.49) µmol/L and LA50 of 1.92 (1.67-2.78) µmol/kg. The toxic effects of these metals were increased when tested in combination with extracts of dissolved organic carbon (DOC) from marine and freshwater sources.

From studying larvae chronically exposed to Pb (60 µg/L), Zn (139 µg/L), Cu (6 µg/L) and Ni (47 µg/L) it was apparent that these metals rendered their toxic effects, at least in part, through disruption of Ca homeostasis. Unidirectional Ca uptake rates as well as Ca ATPase activity were significantly inhibited at various time points over development, in larvae in these metal exposures. This resulted in significantly lower levels of Ca accumulated in the larvae. Interestingly, larvae showed some capacity for recovery as Ca uptake rates and internal Ca levels returned to control values periodically over development. Also metal initially accumulated in the larvae often returned to control levels at 72 h of development with the exception of Pb, which was the only non essential metal out of the four tested. Metal effects on the whole body levels of three other ions (Na, K, Mg) as well as larval weight were modest. Surprisingly, acute exposure to much higher levels of these same metals at various stages during development had negligible inhibitory effects on unidirectional Ca uptake rate, suggesting a mechanism other than direct competition for the Ca uptake sites. We propose studying the toxicity of contaminants periodically over development as an effective way to detect sub-lethal effects, which may not be displayed at the traditional endpoint of 72 h.

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THESIS ORGANIZATION AND FORMAT

This thesis is organized in a sandwich format, as recommended and approved by members of my supervisory committee. It consists of five chapters. Chapter one is a general introduction and overview of background material and the objectives of work. Chapter two, three and four consist of discrete manuscripts that are in preparation for submission to a peer-reviewed scientific journal. The final chapter (chapter 5) summarizes main findings and places these findings in the context of current knowledge.

Chapter 1: **General introduction**

Chapter 2: **Early Developmental profile of the purple sea urchin *S. purpuratus***

Authors: Margaret S. Tellis, Marianna M. Lauer, Sunita Nadella, Adalto Bianchini and Chris Wood

Date of planned submission: May, 2012

Comments: This study was conducted by MST under the supervision of CMW. ML provided technical assistance with animal sampling. SN provided technical assistance. AB provided assistance in developing the methods for the study.

Chapter 3: **The Effect of Pb and Zn on the embryonic and larval stages of development of the purple sea urchin, *S. pupuratus***

Authors: Margaret S. Tellis, Marianna M. Lauer, Sunita Nadella, Adalto Bianchini and Chris M. Wood

Date of planned submission: May, 2012

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Chapter 4: **The Effect of Cu and Ni on the embryonic and larval stages of development of the purple sea urchin, *S.***

pupuratus

Authors: Margaret S. Tellis, Marianna M. Lauer, Sunita Nadella, Adalto Bianchini and Chris M. Wood

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LIST OF ABBREVIATIONS

EC50 - Median effective concentration
LA50 – Median lethal accumulation
NOEC – No observed effect concentration
DOC – Dissolved organic carbon
ASTM – American Society for Testing and Materials

CHAPTER 1: BACKGROUND AND APPLICATIONS OF THE STUDY

Pb, Zn, Cu and Ni in the environment

Inorganic compounds such as metals exist naturally in the environment at low levels. Some, including copper (Cu), zinc (Zn), selenium (Se), are essential for life in small concentrations, but are toxic at higher concentrations. Other metals such as mercury (Hg), cadmium (Cd) and lead (Pb) are classified as xenobiotics with no role in the biological processes of organisms. While natural geological processes such as erosion and volcanism, mobilize metals from the Earth's crust, human activity introduces excess amounts of metals to the environment and raises environmental concentrations many fold above background levels (Wood, 2012).

The metal mining industry in Canada currently accounts for approximately 3.2% of the nation's Gross Domestic Product (GDP) and continues to grow. However industrial activity has also resulted in wide-scale metal contamination of aquatic systems especially at sites close in proximity to industrial waste output (Ringwood, 1992 and Watzin and Roscigno, 1997). The large increases in metal concentrations in the environment over the past century reflect booming industrial activity, which resulted in increased emissions of metal-rich aerosols from smelters and automobile exhausts being brought down to land by precipitation (Schaule and Patterson, 1981). Levels of these metals in oceans are found to be anthropogenetically elevated many-fold above natural levels, in certain areas (Schaule and Patterson, 1981). Once deposited in the environment, metals exist as persistent contaminants due to their conservative nature (inability to be degraded) and environmental concentrations are continuously increasing (Bradl, 2005).

Levels of Pb in natural seawater are normally under 1 μg/L (Brugmann, 1981), but have been reported to be up to 3-200 μg/L in polluted seawater (WHO, 1972). A large source of Pb in fresh or seawater bodies is from the atmospheric deposition of by-products of fossil fuel combustion. The world experienced a significant rise in environmental Pb concentrations following the dawn of the industrial revolution in the latter part of the 20^{th} century. This was in large part due to Pb being used as an additive in gasoline. Since then, the introduction of unleaded gasoline has aided in curbing the addition of Pb to the environment, however excess Pb in the aquatic environment remains a cause for concern (Sánchez-Marín, 2010). Distribution of Pb in the oceans, follows a scavenged-type vertical profile with the amount of dissolved Pb increasing with depth and high surface water concentrations, likely as a result of atmospheric input (Libes, 2009).

Zn concentrations have been measured as high as 1000-10,000 μg/L near sewage treatment plant outflows, exceeding natural levels by up to 2000 times (Eisler, 1993). Distribution of Zn in the oceans follows a nutrient-type profile, with assimilation of the metal into biogenic molecules and phytoplankton, resulting in low surface levels and increasing concentrations with depth due to sinking particles decomposing or undergoing

dissolution.

Cu concentrations are in the tenths to hundreds of ng/L (Sadiq, 1992) in natural seawaters and have been reported to be as high as 29 µg/L (McConchie et al., 1988) in polluted seawaters, which results in a variety of deleterious effects in exposed organisms (Warnau, 1996). Cu distribution in the oceans is intermediate between a scavenged and nutrient-type profile.

Ni Levels are around 0.2 to 0.7 µg/L in open ocean but can reach upwards of 50 to 2000 ug/L in industrially contaminated waters (Pyle and Couture, 2012). Common sources of Ni pollution include industrial, urban and agricultural activities as well as metal mining (Foy et al., 1978). Similar to Zn, Ni follows a nutrient-type distribution in the ocean.

Current Water Quality Criteria (WQC) for Pb, Zn, Cu and Ni vary across the globe in terms of the chemical and biological parameters (ie. water chemistry and life stage) on which they are based (Grosell, 2012; Hogstrand, 2012; Pyle and Couture, 2012 and Mager, 2012). While WQC in freshwater have been well established in various nations, few nations have developed marine WQC for Pb, Zn, Cu and Ni (Grosell, 2012; Hogstrand, 2012; Pyle and Couture, 2012 and Mager, 2012). Current marine WQC for Cu, Zn, Pb and Ni for various nations are as follows: Cu WQC in USA, EU and Australia/New Zealand range from 1.3– 4.7 µg/L (Grosell, 2012). Zn WQC of USA and Australia/New Zealand range from 15 to 85.6 µg/L (Hogstrand, 2012). Ni WQC in Australia/New Zealand and as determined by the World health Organization range between 1 and 20 µg/L (Pyle and Couture, 2012). Pb WQC in South Africa, USA and Australia/New Zealand, range from 4.4 – 12 µg/L (Mager, 2012). Unfortunately Canada, with the exception of British Columbia, is yet to establish WQC for any of these four metals in the marine environment (Grosell, 2012; Hogstrand, 2012; Pyle and Couture, 2012 and Mager, 2012). In British Columbia the marine WQC for three out of the four metals are as follows: Pb - 2 µg/L, Zn - 10 µg/L and Cu - 2 µg/L (Retrieved from Government of British Columbia Environmental Protection Division website: http://www.env.gov.bc.ca/wat/wq/wq_guidelines.html)

Toxicity of Pb, Zn, Cu and Ni

According to Wright and Welbourn (2002) Pb, Cu and Ni are metals of major environmental concern. Pb and Ni are also classified as priority contaminants in European Union regulations on water policy as they are both toxic to man as well as aquatic organisms, have the capacity to bioaccumulate and biomagnify, and also cause damage to DNA (European Commission, 2001).

Freshwater studies have shown Pb and Zn to have a variety of effects on reproduction (Berglin et al., 1985), behavior, growth and survival (Eisler, 1993) in a number of invertebrate species. In part, Pb renders its toxic action through an ability to mimic Ca and disrupt fundamental Ca^{2+}-dependant physiological processes as seen in freshwater fish (Rogers et al., 2004). In fish, Pb disrupts Na^{2+}, Cl^{-} and Ca^{2+} homeostasis during acute exposure and hemoglobin synthesis during both acute and chronic exposure

(Rogers and Wood, 2004; Rogers et al., 2003; Hodson et al., 1978). As an essential ion, Zn plays a role in over 300 enzymes and proteins (Vallee and Falchuck, 1993) and is an important constituent of enzymes regulating meiosis in developing sea urchin larvae (Waurnau et al., 1996). However, Zn is deleterious to organisms at higher levels. Similar to Pb, Zn also disrupts Ca homeostasis in freshwater fish through the induction of hypocalcaemia as well as an offset of acid base balance (Spry and Wood, 1985).

Cu is also an essential trace element, found in many key biological molecules such as hormones, vitamins, many enzymes and nucleoprotein complexes (Phillips, 1977). Due to its importance in several biological processes, Cu levels in organisms are strictly regulated. However, higher levels of Cu are introduced to animals through environmental contamination. At these levels, Cu proves to be toxic and can act as a biocide (Warnau et al, 1996). The impact of Cu on the aquatic system is a grave concern as fish, crustaceans and algae exhibit 10 to 1000 times greater sensitivity to Cu than mammals (Wright and Welbourn, 2002). Interestingly, of the common metals, Cu has the narrowest margin between essentiality and toxicity (Sánchez-Marín, 2010). Fish chronically exposed to Cu suffer various consequences such as a loss of appetite, growth suppression, ionoregulatory disturbance, lower aerobic capacity and higher mortality (Taylor et al., 2000). Copper has also been known to disrupt ionic balances by altering the activity of membrane-located carrier proteins or ATPases (Li et al., 1996) as well as carbonic anhydrase (Zimmer et al., 2012).

Ni is another essential ion, which becomes toxic to organisms at higher levels. It is also a known Ca homeostasis disruptor, acting antagonistically against Ca and blocking several different types of Ca channels (Lee et al., 1999). Additionally Ca has been observed to be protective against Ni exposure in rainbow trout (*Oncorhynchus mykiss)*, fat head minnows (*Pimephales promelas)* and the water flea (*Daphnia pulex)* (McFarlane and Gilly, 1998; Todorovic and Lingle, 1998 and Deleebeeck et al., 2007). Ni also acts as an antagonist to Mg in many mammals, birds, bacteria and fungi (Eisler, 1998). The exact mechanisms of Ni transport in cells are not completely known however, Ca^{2+} and Mg^{2+} channels have been identified as possible routes of entry (Eisler, 1998).

Invertebrate embryos often have large Ca requirements to support increased demands for rapid development and formation of structures such as mussels' shells or sea urchins' spicules. Therefore, the toxic effects of Pb, Zn, Cu and Ni exposure on ionic balance as well as Ca-mediated pathways may be particularly detrimental at the embryonic stage where normal calcium homeostasis is vital for rapid development.

While extensive research exists on the effects of Pb, Zn and Cu in freshwater environments (Deleebeeck et al., 2007; Kozlova et al., 2009), there is limited research on the effects of Pb, Zn and Ni in marine and estuarine environments (Supanopas et al., 2005; Franca et al., 2005; Kobayashi & Okamura, 2004; Radenac et al., 2001). There are even fewer studies examining the effects of environmental modifiers such as salinity and dissolved organic carbon (DOC) on the toxicity of Pb and Ni (Sánchez-Marín, et al., 2007a and 2007b; Nadella et al, 2009), and to our knowledge none on Zn in marine organisms.

DOC is a surrogate measure of dissolved organic matter (DOM), the broad name

given to various dissolved organic molecules derived from decaying organic matter. High levels of DOC are generally protective to sea urchin embryos as the DOC molecules form strong inert complexes with metals, thus reducing their bioavailability (Sánchez-Marín et al., 2007a). They vary in protection depending on their sources and fulvic acid (FA) relative to humic acid (HA) content. HAs contain carboxylic and phenolic groups which are known to bind to metals such as Cu and Pb (Sánchez-Marín, 2007a). Although DOC is quite protective to freshwater fish (McGeer et al., 2002; Niyogi and Wood, 2004), there is still much uncertainty about the effects of these organic molecules on Pb, Zn, Cu and Ni toxicity in marine environments (Al-Reasi et al., 2011).

The oceans are of great social and economic significance, but there is a paucity of toxicity data on the marine environment. Extensive research is required to fill this void in our knowledge, as one cannot rely on the precautionary principle alone when determining water quality guidelines.

Development of water quality guidelines

The precautionary principle, as articulated at the Wingspread Conference in Racine, Wisconsin (1998) states that "When an activity raises threat of harm to human health or the environment, precautionary measures should be taken even if some cause and effect relationships are not established scientifically". Put simply, extreme caution should be exercised first and scientific justification can follow (Appell, 2001). This is not a preferred method of risk assessment as there is a large degree of uncertainty intrinsic to this approach. Policy makers have a responsibility to strike a fair medium between being excessively protective in imposing unreasonable restrictions on industry and being not cautious enough, thereby allowing major damage to be inflicted on the aquatic environment (Wood, 2012)

Metals toxicity research reduces uncertainty surrounding regulations and provides scientific data on which regulators and risk assessors can base decisions. An important factor in reducing uncertainty is to account for the influence of all environmental factors on toxicity. In recent years the biotic ligand model (BLM; DiToro et al., 2001, Santore et al., 2001) has gained more popularity as a realistic model for predicting toxicity of contaminants. The BLM incorporates both abiotic factors such as water chemistry as well as biotic ligands such as the organism's receptors for toxicants. This approach calculates critical accumulation of dissolved metal at the site of toxic action using commonly measured water parameters such as temperature, dissolved organic carbon (DOC), pH, alkalinity, salinity, Ca, Mg, Na, K, SO_4^{2-}, and Cl^- (USEPA, 2006). The model is not a complete solution to the challenges of deriving water quality regulations, but it provides more scientifically sound reasoning for environmental guidelines as well as advances our knowledge of toxic action of contaminants. This reduces the uncertainty of the precautionary principle and the potential risk of environmental damage (DiToro et al., 2001, Santore et al., 2001). Attempting to elucidate the mechanisms of toxic action of these metals also helps reduce uncertainty in the development of guidelines. Mechanisms of toxicity, such as sub-lethal effects on molecular and biochemical processes are important factors to consider when developing policy. They provide a more sensitive,

refined endpoint, which can be detected earlier and at lower concentrations than traditional endpoints such as mortality.

The Canadian approach to developing and applying environmental quality guidelines incorporates three fundamental principles. The first is that environmental quality should be maintained so that no observable effects on aquatic or terrestrial ecosystems are sustained in the long term. This involves conservative assumptions, which attempt to ensure the protection of the most sensitive species and life stages. The second principle is that guidelines should be developed for major uses of land and water (i.e. resource-based use). This includes areas that are used for recreation and agriculture and takes into consideration social and economic impacts. The last principle is that guidelines must be based on extensive scientific data, which takes into account site specificity (Gaudet et al., 1995).

The current research encompasses all three guiding fundamental principles. In studying toxicity of metals to the larval and embryonic stages of the Pacific Purple Sea Urchin (*Strongylocentrotus purupuratas*) we are studying a very sensitive organism at its most sensitive stage of life. Protection of the most sensitive species by default provides protection to a whole host of more robust organisms at all sensitive life stages.

Over the decades the sea urchin has been a highly used sensitive test organism in toxicity experiments routinely performed to monitor pollutants in marine environments (Tabata, 1956; Okubo and Okubo, 1962; Kobayashi, 1971, 1980, 1994). The Pacific Purple Sea Urchin (*S. purpuratus*) was therefore an obvious choice for our work as there is an existing wealth of knowledge on this test organism, upon which to build.

Test organism

Sea urchins belong to the Phylum Echinodermata, Sub phylum Echinozoa and Class Echinoidea. They are largely referred to as echinoids. The phylum exhibits a wide spread marine distribution across the globe and approximately 6000 species have been discovered to date. Seven of these species are present along the marine coasts of Canada (Environment Canada, 2011).

Echinoids are complex invertebrates with a fairly advanced structure and a variety of complex features. They share many similarities with chordates including certain biochemical process and their essential pattern of embryonic development. Additionally, the five sections of their body, which are organized in a radial fashion around a central axis are said to be superimposed on a bilateral organization (Environment Canada, 2011). Their distribution (from the Pacific coast of Canada down to Baja California) (Meinkoth, 1981) and long periods of gravidity (January to March for feral animals, October to April on the Californian coast) make them a convenient test organism applicable to many regions (Environment Canada, 2011).

Early development of the sea urchin (fertilization to the pluteus larval stage), has been of great interest in the field of embryology with over 5000 papers being published on the subject by 1981 (NRC, 1981). This wealth of knowledge has led to sea urchins being a widely used organism for toxicity tests over the years (Lillie, 1921 and Bougis, 1959).

Embryonic and Larval bioassays

The early embryonic and larval stages of marine organisms are important life stages in which to examine metal toxicity due to increased sensitivity experienced at these times in comparison to their adult counterparts (His et al., 1997; Novelli et al., 2003). Viability of embryos is also a crucial factor when estimating later adult population sizes (Gosselin and Qian, 1997). Larval and embryonic bioassays are relevant and useful tests to determine toxicity to aquatic organisms as effects are observed in hours or days. From a practical standpoint these tests are economical, sensitive, scientifically valid and yield rapid results. Bioassays also enable us to determine realistic toxicity of contaminants through studying interactions of contaminants with organisms. Metals in particular interact at a cellular level by competing for cell membrane binding sites (e.g. ion-transport proteins), metallo-enzymes and metallothioneins, resulting in effects that are not purely additive (Sharma et al., 1999).

Initial work from our laboratory has focused on elucidating threshold toxicity values for these metals (EC_{50} and LA_{50}). The EC50 or median effective concentration, is the concentration of a substance that causes 50% of a larval test sample to be morphologically abnormal, relative to controls. The LA50 or lethal accumulation concentration is the amount of metal accumulated within the test subjects associated with 50% abnormality in the larvae. While findings from this work provide valuable information about the toxicity of Pb, Zn, Cu and Ni, they are limited in that they are based purely on survival and morphological criteria (i.e. occurrence of deformities) and as such only provide information on a macroscopic scale. To delve further into the molecular mechanisms of toxic action of metal pollutants, a novel approach was undertaken in the current thesis to assess toxicity at different stages of development.

Developmental stages of S. purpuratus in the first 96 h following fertilization

Sea urchin embryos have been long used as a model system for analyzing cellular activities during early development owing to their rapid differentiation, small number of constituent cells and simple organization (Kominami and Takata, 2008). For the purposes of the current research, the rapidly developing sea urchin is an ideal test organism as it allows us to study the effects of these metals on different cellular processes as they appear in the developing embryo. This is an effective way of pinpointing mechanisms of toxicity.

According to Kominami and Takata (2008), upon fertilization the embryo rapidly divides through radial and holoblastic cleavages for the first 1-1.5 hrs. Cleavage is equal and cells are identical until the embryo reaches the 8 cell (blastomere) stage after which nonsymmetrical cleavage starts to take place. The embryo undergoes 7 rounds of synchronous cleavage every 0.5-1 h after which synchrony of division is lost. By 24 h the fertilized egg has undergone 10 cleavages and has developed into a hollow ball called a blastula. This is followed by gastrulation. At this stage the embryo develops three germ layers – mesoderm, endoderm and ectoderm. Gastrulation also marks the initiation of skeletogenesis in which the skeletogenic primary mesenchyme cells are deposited and begin to form the spicule. After gastrulation, the embryo undergoes organogenesis and develops into the pluteus larvae at approximately 72 h (Figure 1).

It is clear that embryonic and larval stages of development are a very rapidly changing phase in the sea urchin's life, where cellular make-up and activities may differ vastly from hour to hour. Accumulation, utilization as well as "permeability" to Ca during the course of development are therefore highly variable over time (Nakano et al., 1963). In turn one would expect the extent to which metal pollutants disrupt fundamental Ca-dependant processes to then also vary over development. This underscores the need for examining metal toxicity at regular time intervals over development.

Objectives of this study

Initial research focused on investigating the ionoregulatory physiology of normal sea urchin embryonic and larval development, as it was suspected that some of the processes would be targets for metal toxicity. This data was meant to aid in the overarching goal of elucidating the mechanisms of toxicity of Pb, Zn, Cu and Ni to developing sea urchin embryos and larvae.
To fulfill this goal my specific objectives were:

1) To investigate the early developmental Ca profile of sea urchin embryos and larvae during the first 96 h of development.
2) To determine patterns in accumulation of other major cations (Mg, K and Na) in the early stages of embryonic and larval growth
3) To determine median threshold values (EC50 and LA50) for Pb and Zn as well as the relative ionoregulatory disturbances associated with the varying amounts of metal accumulated in the larvae.
4) To determine the salinity threshold for developing sea urchin larvae
5) To explore the synergistic effects of salinity and DOC on Zn toxicity
6) To develop a comparison of the different stages of the first 96 h of development. Here, the basic hypothesis was that stages which exhibit the most pronounced changes will be especially vulnerable targets of metal toxicity.
7) To determine the effects of Pb, Zn, Cu and Ni on the Ca uptake profile during the first 96 h of development.
8) Investigate whether ionoregulatory disruption is a mechanism of toxicity of Pb, Zn, Cu an Ni through measuring ions over development in exposed larvae as well as over a range of concentrations of metals at the 72 h time point

Our findings, which fulfill the preceding objectives, are outlined in the three experimental chapters of this thesis. In the first chapter, unidirectional Ca uptake experiments, Ca ATPase activity and Ca accumulation were measured and through this data we were able to determine the early developmental profile of Ca in developing sea urchin embryos. It was discovered that Ca uptake is variable over development and depends on the requirements of the developing sea urchin at the specific time point. Ca ATPase and Ca accumulation followed the same pattern as Ca uptake, however there was a lag period in between increased Ca uptake and resulting increases in amount of Ca accumulated. Levels of other ions (Na, K and Mg) were also variable over time as the developing larvae presumably altered net uptake of these electrolytes depending on its

developmental requirements. However, larval weights did not differ significantly over development until the 96 h time point at which time their weight increased significantly. From our various measurements, the gastrulation stage proved the most dynamic phase of the 96-h development period, characterized by large increases in Ca uptake, Ca accumulation and Ca ATPase activity.

In the second and third chapter the objective of elucidating the toxic mechanisms of Pb, Zn, Cu and Ni was met through analyzing the same biomarkers as the first chapter, but in larvae chronically exposed to the respective metal over the development period. Unidirectional Ca uptake was significantly inhibited by metals during stages when Ca uptake was highest. This ultimately resulted in lower Ca accumulated in exposed larvae. The gastrulation period was most sensitive to metal exposure, and exhibited the most pronounced Ca uptake inhibition in all metal exposed larvae. Surprisingly, these inhibitions of Ca uptake occurred for all four metals during chronic exposure, but generally did not occur when developing larvae were acutely exposed to much higher metal levels at various stages. This suggests that the metals are acting by a mechanism(s) other than direct competition for the Ca uptake site. Threshold values for developing urchins were also determined. Embryos displayed a very low threshold for changes in salinity with less than 80% of the embryos surviving in 90% seawater. Median threshold values (EC50 and LA50) were determined for Pb and Zn using standard ASTM protocol. EC50 protocol is summarized in Figure 1.2 (ASTM, 1994). The EC50 for Zn was 151 (129-177) µg/L and the LA50 was 316 (141-741) µg/g. The EC50 for Pb was 74 (51-101) µg/L the LA50 was 398 (347-575) µg/g.

In general, ionoregulatory disturbance was caused by levels of metal exposure that were lower than the respective EC50s for each of these metals. Metal exposure concentrations, which invoked effects of toxic stress were also not much higher than the WQC values reported earlier in this chapter, especially in the case of Cu where effects were seen during a chronic exposure of 6.15 µg/L where as the WQC for the European Union is 4.7 µg/L (Grossell, 2012). Effects of these metals were also observed at early stages in development and were often reversed before the 72 h time point. This is of significance as 72 h is the standard endpoint used in toxicity testing. Our research suggests that a more accurate measure of toxicity can be attained, by measuring biomarkers of toxicity at regular intervals prior to the 72 h time point.

Figure 1-1 General early developmental stages of sea urchins. Note this figure was adapted from a figure created by B. E. Stavely for Molecular & Developmental Biology (BIOL3530), Department of Biology, Memorial University of Newfoundland. http://www.mun.ca/biology/desmid/brian/BIOL3530/DB_Ch06/fig6_19.jpg

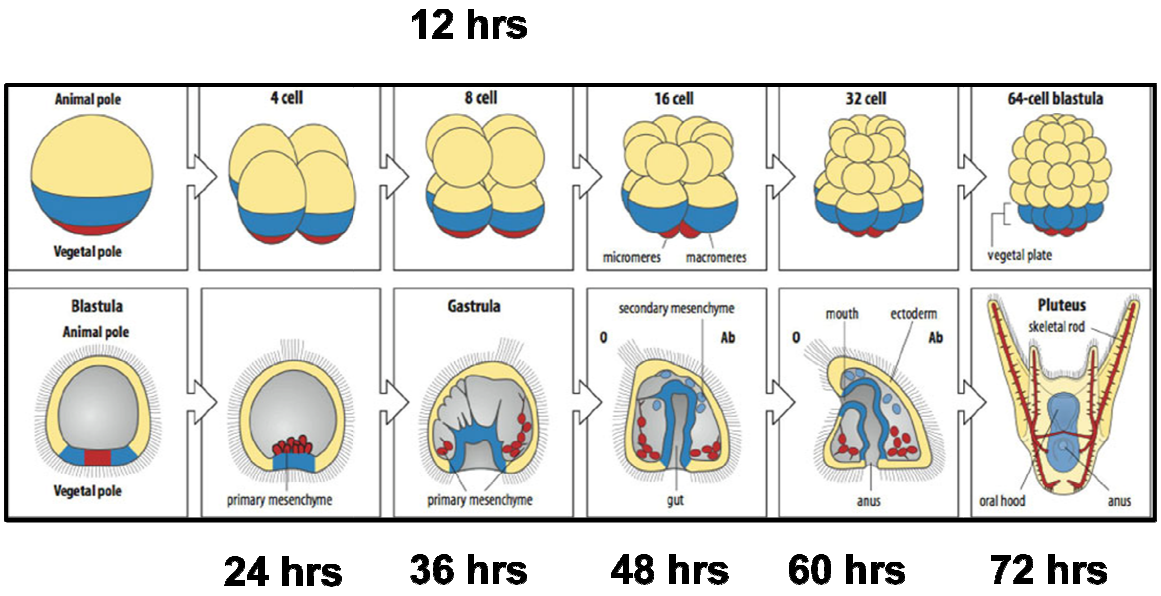
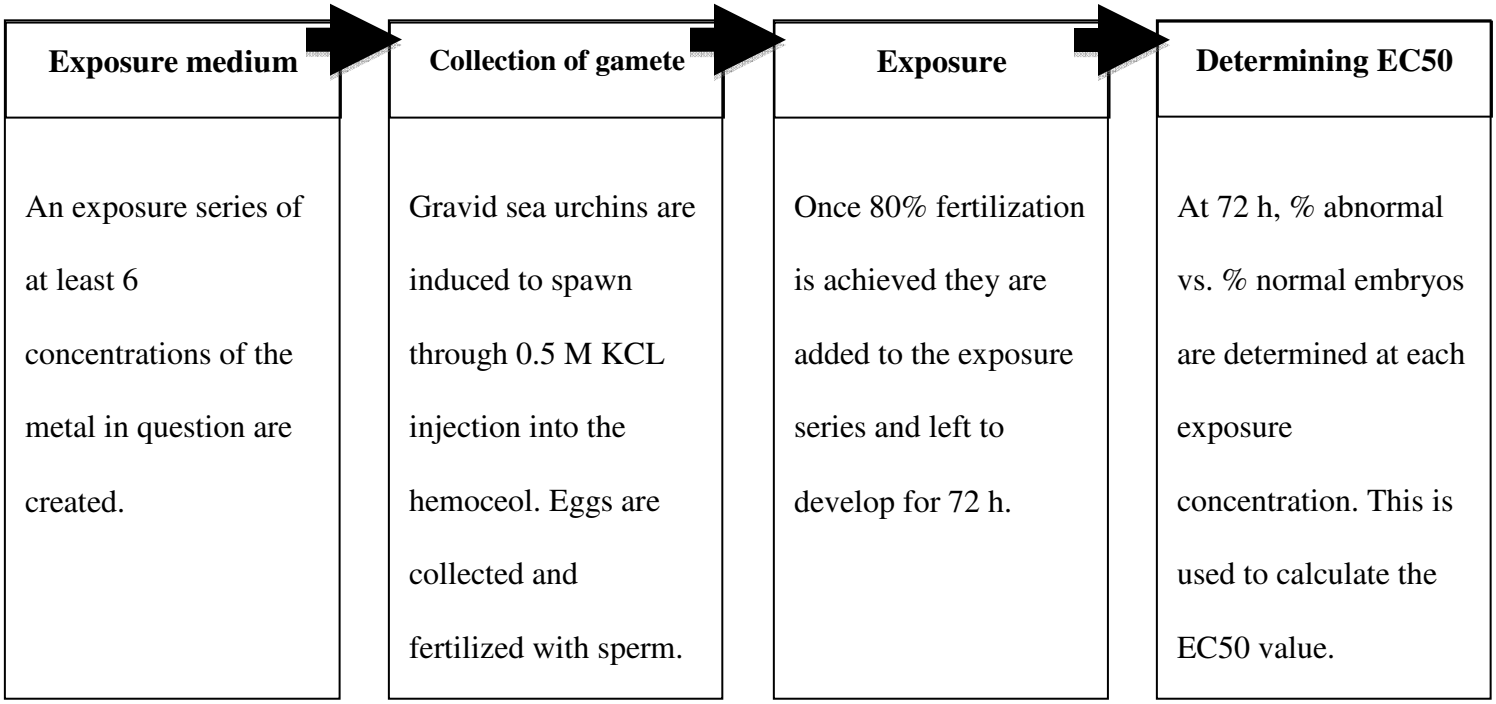

12 hrs
Animal pole
Vegetal pole
4 cell
8 cell
16 cell
micromeres
macromeres
32 cell
64-cell blastula
vegetal plate
Blastula
Animal pole
Vegetal pole
primary mesenchyme
Gastrula
primary mesenchyme
secondary mesenchyme
O
Ab
gut
mouth
ectoderm
O
Ab
anus
Pluteus
skeletal rod
oral hood
anus
24 hrs
36 hrs
48 hrs
60 hrs
72 hrs

Figure 1-2 A flowchart summarizing the standard ASTM method (1994) used to determine EC50 concentrations .


Exposure medium
An exposure series of at least 6 concentrations of the metal in question are created.
Collection of gamete
Gravid sea urchins are induced to spawn through 0.5 M KCL injection into the hemoceol. Eggs are collected and fertilized with sperm.
Exposure
Once 80% fertilization is achieved they are added to the exposure series and left to develop for 72 h.
Determining EC50
At 72 h, % abnormal vs. % normal embryos are determined at each exposure concentration. This is used to calculate the EC50 value.

CHAPTER 2: THE EARLY DEVELOPMENTAL PROFILE OF THE PURPLE SEA URCHIN (*S. PURPURATUS*)

Abstract

In order to investigate the early developmental profile of purple sea urchin (*S. purpuratus*), whole body Ca uptake, ion content, Ca ATPase activity and growth were examined over the first 96 h of development. Ca uptake rates were highly variable over the early developmental stages, increasing during the periods of blastulation to gastrulation, then declining until their rise again late in the pluteus stage. CaATPase activity was low during initial cell cleavages at 12 h but increased from blastulation until the pluteus larval stage at which stage measured activity was low again. Whole body Ca content increased steadily through to the pluteus stage, but Mg, K and Na contents were variable in their pattern over development. Larval weights did not change significantly for the majority of the 96 h development but were significantly higher at the 96 h. Overall, the gastrulation stage of development displayed the most pronounced changes, as Ca uptake and accumulation and Ca ATPase levels were the highest at this stage compared to any other stage over the first 96 hours of development.

Introduction

Early embryonic and larval development of the sea urchin has been of great interest in the field of embryology with over 5000 papers being published on the subject by 1981 (NRC, 1981). Characteristics of sea urchin embryos such as their transparency, rapid differentiation and simple organization of constituent cells, have made them an ideal test organism for biochemical and molecular studies resulting in them becoming a model system for analyzing cellular activities during early development (Kominami and Takata, 2008).

Years of extensive research have led to a detailed understanding of the early stages of development of the embryo. Upon fertilization the embryo rapidly divides through radial and holoblastic cleavages for the first 1-1.5 h. Cleavage is equal and cells are identical until the embryo reaches the 8 cell (blastomere) stage, after which nonsymmetrical cleavage starts to take place. The embryo undergoes 7 rounds of synchronous cleavage every 0.5-1 h until synchrony of division is lost. By 24 h the fertilized egg has undergone 10 cleavages and has developed into a hollow ball called a blastula. This is followed by gastrulation. At this stage the embryo develops three germ layers – mesoderm, endoderm and ectoderm, and a rudimentary gut (Kominami and Takata, 2008). Gastrulation also marks the initiation of skeletogenesis in which the skeletogenic primary mesenchyme cells are deposited and begin to form the calcium carbonate skeleton, commonly referred to as the spicule (Farach et al., 1987). After gastrulation, the embryo undergoes organogenesis and develops into the pluteus larvae at approximately 72 h (Kominami and Takata, 2008) (Refer to Figure 1-1).

Of the various developmental events, the formation of the spicule is of great interest with extensive research being performed on its synthesis (Decker and Lennarz, 1988; Wilt, 1999; Harkey et al., 1995). Larval uptake of ions is upregulated during this time, with calcium in particular playing a pivotal role in the biomineralization of the spicule (Wilt, 1999). This developmental stage is therefore of great importance from an ionoregulatory perspective. Echinoderm larvae in general are very poor ionoregulators and are viable only within a narrow margin of salinities (Kinne, 1971). Within this narrow range, developing urchin embryos are able to maintain ion homeostasis by modifying cell membrane potential, which regulates cell permeability to ions. Ca influx for instance is mediated by voltage-gated channels (de Araújo Leite and Marques-Santos, 2011). Movement of ions into the embryo through transporters and voltage-gated channels is highly influenced by the concentrations of ions in the external medium (Hagiwara and Jaffe, 1979) as well as the developmental requirements of the sea urchin embryo (Payan et al., 1981).

Developing sea urchins have a particularly high requirement for Ca over development, as it has important physiochemical roles in cell division (Heilbrunn, 1943; Hultin, 1950; Gross, 1954) and in the form of Calcium Carbonate ($CaCO_{3)}$ is the predominant component of the spicule (Wilt, 1999). Ca homeostasis over development is therefore of particular importance and fundamental processes involving Ca transport and accumulation are of interest to study. As such, Ca homeostasis will be the focus of our research.

In this chapter we aim to investigate ionoregulatory physiology of normal development as we suspect that some of these processes may be targets of metal toxicity. To achieve our objective we will be studying the Ca profile of early development by assessing Ca uptake and accumulation as well as measuring Ca ATPase activity over development. We will also be monitoring absolute whole body concentrations of ions over development to assess the ionic requirements of each developmental stage.

Materials and methods

Experimental organisms

Reproductively ripe adult sea urchins (*S. purpuratus)* were obtained by scientific diving teams, from the natural benthic populations of Barkley Sound, B.C., Canada (48°50'30" N, 125°08'00" W). The sea urchins were held in aerated tanks supplied with flow-through seawater (32 ppt) at 15 ˚C to keep gonads ripe and prevent premature spawning or reabsorption of gametes. Only minimal handling of the urchins was permitted so as not to inflict unnecessary stress on the brood stock.

Collection of gametes

Spawning was induced through an injection of 1 ml of a 0.5 M KCl solution into the haemocoel of the adult sea urchin following a method described by Hinegardner (1975). Spawning females were placed upside down on 50 ml Falcon™ tubes containing filtered (0.2 µm Steritop™ filter– Millipore, Billerica, MA, USA) seawater (100%), into which the eggs were collected. The eggs from different females were then pooled by filtration through a mesh into a single beaker. Filtration removed any debris or detritus from the egg solution. A drop of sperm from the spawning males was diluted in 50 ml of filtered (0.2 µm) seawater. 1 ml of this diluted sperm was then added to the pooled sea urchin eggs to initiate fertilization. The solution was gently stirred periodically to facilitate fertilization, which was normally achieved in under 0.5 h. Fertilization success was determined under a microscope, through the appearance of a fertilization membrane around each egg. Once fertilization of 80% of the eggs was achieved, the egg solution was agitated gently to ensure homogeneity of eggs in suspension and the density of eggs in 1 ml of the egg solution was determined under the microscope. The egg solution was diluted using filtered seawater until the desired concentration of eggs in the stock solution was obtained. The egg stock solution was required to be at a density which would result in a final concentration of 1200 eggs/ L exposure solution. After the embryos were added to each replicate of exposure solution they were then allowed to develop in an incubator at 15 °C with 14 h light: 8 h dark light cycle for the desired time of each test. The larvae were sampled every 12 h in order for a variety of endpoints to be analyzed periodically over early development.

Radioactive ^{45}Ca uptake rate measurements

Unidirectional uptake rates of Ca from the water, as determined by ^{45}Ca

incorporation, were measured in separate batches of embryos at 12-h intervals over the first 96 h of development. For each flux rate determination, embryos were sampled from each replicate by gently stirring it to ensure homogeneity of embryos in suspension. Then a small volume was removed from each of the 6 replicates. The extracted volume was decreased to a few ml by filtering out some of the seawater by gravity using a Steritop™ filter (0.2 µm – Millipore, Billerica, MA, USA) leaving a concentrated embryo solution with a nominal target density of 2500 embryos/ml. ^{45}Ca uptake rate measurements were then performed on the embryos.

Ca flux rate measurements were performed by incubating 0.5 ml of sea urchin embryos suspension with 0.5 ml of radioactive ^{45}Ca (0.17 µCi/ml as $CaCl_2$, PerkinElmer, Woodbridge, ON, Canada) in seawater in 2-ml Eppendorf™ tubes for 20 min. The flux period was based on preliminary time series experiments in which 20 min was determined to be optimal for ^{45}Ca uptake analysis (data not shown).

On completion of the 20-min flux period, the larvae were removed from the Eppendorf™ tube via a 1-ml syringe. A 45-µm syringe tip filter (Nalgene™ Rochester, NY, USA) was then attached to the end of the syringe and the flux solution held in the syringe was injected through it, leaving the embryos on the filter. Then 10 ml of fresh seawater was immediately passed through the filter to wash the larvae. The filter was then reversed and 3 x 1 ml (i.e. each ml separately) of fresh seawater was passed through the filter and collected in a scintillation vial to recover the embryos. 5ml of scintillation fluid (ACS, GE Healthcare - Piscataway, NJ, USA) was added to each vial and the ^{45}Ca in the sample was measured using a beta counter (Tm Analytic, Beckman Instruments, Fullerton, CA, USA). A dummy run (without ^{45}Ca) of the flux procedure was performed at each time point and the embryos recovered from the filter were counted under the microscope to determine embryo concentrations used in the flux measurements.

Unidirectional Ca uptake rates were calculated from the counts per minute of each replicate (CPM), mean specific activity (SA), number of larvae in each replicate, and experimental time (t), and were expressed as pmol Ca/larvae/h:

Ca uptake = (CPM/SA) * (1/# of larvae)*(1/t)

Note: Specific activity was calculated by dividing the Ca (pmol/ml) in seawater by ^{45}Ca radioactivity (CPM/ml).

ATPase enzyme analysis

At each 12-h time point, 5 replicates of each exposure (control, Pb and Zn) were sampled. The volume of each replicate was reduced to 20 ml through filtering out exposure solutions by gravity through a 0.45 µm filter (Nalgene™ Rochester, NY, USA). The concentrated embryos were resuspended in fresh seawater and concentrated again. This was performed 3 times to wash the larvae. The washed, concentrated larvae were then centrifuged at 12000 g for 5 min, the supernatant was decanted and the resulting pellet was transferred to an Eppendorf™ tube and frozen at -70 °C for later analysis.

Ca^{2+}ATPase analysis was performed through a method which measured liberation of inorganic phosphate by the ATPase enzyme (Vijayavel et al., 2007).

Ca^{2+}ATPase activity was determined as the amount of inorganic phosphate per mg of protein per hour released in a medium containing 80 mM NaCl, 5 mM $MgCl_2$, 3 mM ATP, 20 mM Tris–HCl (pH 7.4), 0.5 mM $CaCl_2$ and 1 mM ouabain.

Ion analysis

Every 12 hours over the first 96 hours of development, 5 replicates were sampled. The embryos in each replicate were filtered via a vacuum pump through a 0.45 µm filter (Nalgene™ Rochester, NY, USA and rinsed with nanopure water. The filter paper was then placed in an open Eppendorf™ tube and left to dry at room temperature. Ion levels (Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) were then measured using flame atomic absorption spectroscopy (220FS; Varian Instruments, Palo Alto, CA, USA). Reference standards used were TM24 and TM25 (Environment Canada certified reference material, recovery 90 %-95%).

The dried filter with collected embryos on it was weighed. This weight minus the filter weight divided by the number of larvae collected was taken to be the mean dry weight of the developing embryo or larvae.

Statistical Analysis

Statistical analysis was performed with SigmaPlot 10.1. One-Way ANOVAs were used to detect variation over time and where the F value indicated significance, Fishers LSD test post hoc tests were used to identify specific significant differences. Prior to test, all data were checked for homogeneity of variances and normality of distribution, and where necessary were transformed using natural logarithm or square root functions. All data are presented as means ± SEM (N, number of replicates) on non-transformed data. Changes were considered significant at $p < 0.05$.

Results

Ca uptake over 96 hours

Unidirectional Ca uptake rates by developing embryos and larvae were variable over the first 96 h of development (Figure 2-1). Uptake was low at 12 h when fertilized sea urchin eggs were undergoing the initial cleavages. However a large increase in Ca uptake rate was observed 12 h later at the mesenchyme blastula stages (~24 h). The next large increase in Ca uptake rates was at gastrulation (~48 h), thereafter Ca uptake decreased and reached a plateau for the next 36 hours but increased slightly again at 96 h when the larvae were in the pluteus larval stage of development (Figure 2-1).

Larval weights

Larval dry weights did not differ until the 96 h time point at which they were significantly higher than all previous time points. (Figure 2-2).

Whole body ion content of larvae over development

Whole body Ca concentrations (based on dry weight) showed interesting patterns

of increase over 96 h of development. Ca concentration was initially low for the first 36 h, after which there was a significant increase in Ca content of the larvae at 60 h. There was another significant increase in larval Ca levels at 72 h of development, after which there was no change in Ca levels for the remainder of the 96 h (Figure 2-3a).

Whole body Na, Mg and K levels (on a dry weight basis) were variable in their accumulation pattern over development (Figures 2-3 b, c, d). K levels in embryos decreased over time until gastrulation at 48 h after which levels increased again (Figure 2-3d). Na levels were constant over development for the most part, but were significantly higher at 24 h as well as during the pluteus larval stage (72 h onwards) Figure 2-3b). Mg levels were also fairly steady over development apart from 24 h and the pluteus larval stages when they were significantly higher (Figure 2-3c). In terms of absolute values, Na was present at concentrations approximately 10 times higher than the other ions measured whereas K, Ca and Mg concentrations were comparable.

Ca ATPase Activity

Whole body Ca ATPase activity increased significantly from low levels at 12 h to a peak at gastrulation (48 h). This was followed by a decrease in activity over the next few hours till low levels of activity at 72 h when the larvae were entering the pluteus larvae stage (Figure 2-4).

Discussion

The findings of this work provide reference values as well as a developmental profile for various biological endpoints over early embryonic and larval growth of the purple sea urchin *S. purpuratus*. The results also pertain directly to refining toxicity bioassays, by illuminating possible biological endpoints and developmental stages to consider in future toxicity tests.

Ca uptake

Ca uptake showed an interesting pattern of variation over the 96 h development. The rate of Ca uptake in sea urchin larvae was extremely low during the initial cell cleavages at 12 h of development (Figure 2-1), a finding consistent with reports from past studies on *P. depressus* (Nakano et a., 1963) and an increase in Ca uptake was first observed at the mesenchyme blastula stage (24 h) after which the next increase was observed at gastrulation (48 h). The increased Ca taken up during the mesenchyme blastula stage is presumably due to an increase in accumulation of Ca in the primary mesenchyme (skeletogenic) cells of the blastocoel, which accumulate Ca to be later deposited on the spicule (Nakano et al., 1963). According to Wilt (2002) Ca is imported and precipitated intracellularly in vesicles in the form of amorphous calcium carbonate (ACC) where it binds with proteins and is thus stabilized. The next increase in uptake we observed at the gastrulation stage is explained by the start of Ca deposition on the spicule, which marks the initiation of skeletogenesis (Orström and Ortsröm 1942; Yasumasu, 1959). At this stage ACC is thought to be exported from the primary mesenchyme cells and deposited on the spicule where it is transformed into calcite (Wilt, 2002). Ca uptake

decreases following gastrulation, as the larvae presumably utilize Ca from stores in primary mesenchyme cells. However, Ca uptake again increases once the pluteus larva is fully formed. It has been extensively reported that although gastrulation marks the formation of the rudimentary gut, the larvae is only ready to feed in the pluteus stage (Hinegardner, 1969). The increase in Ca uptake occurring simultaneously around the time when the larvae are ready to feed leads one to believe that the gut of the larvae could be an additional route of Ca uptake (Figure 2-3). Interestingly, the 96 h time point was the only stage of development where larvae were significantly heavier. This could in part be due to increased Ca uptake and accumulation at this time point (Figure 2-2).

This Ca uptake experiment was repeated in subsequent toxicity experiments (see Chapters 3 and 4) and the same pattern was observed. However the pattern was shifted to later time points during the test period. It is therefore important to note that although the sequence of developmental stages remain consistent, the time scale of development may vary by a few hours between trials as a result of the natural biological variability of test organisms.

Ca ATPase activity

Ca ATPase activity (Figure 2-4) showed loose correlation with the pattern of Ca uptake (Figure 2-1) observed over early development of the sea urchin embryo. Ca ATPase activity was initially low at 12 h and increased at 48 h during the gastrulation phase after which it decreased during the pluteus larvae stage at 72 h. Past studies on Ca ATPase mRNA show similar patterns of increase over time. Gradual increases in Ca ATPase mRNA were observed from the blastula stage and peaked by the mid - to late blastulae stage (Jayantha and Vacquier, 2007). The mRNA in the mentioned study peaked earlier in development than Ca ATPase activity in the present study however appearance of mRNA transcripts often precedes real time expression of the proteins for which they encode.

The pattern of CaATPase activity up to 72 h was similar to that observed with Ca uptake rates. Ca ATPase activity however is not directly responsible for Ca influx as influx is mediated by Ca voltage gated channels (de Araújo Leite and Marques-Santos, 2011). Ca ATPase is involved in compartmentalization of Ca and mitotic apparatus assembly (Mazia et al., 1937). Its activity therefore might be an indicator of embryonic Ca demands over development, which are reflected in Ca uptake rates.

Ca accumulation

Ca was the only one out of the four ions (Ca, K, Na and Mg) measured that increased consistently in whole body concentration over the 96-h development period (Figure 2-3 a). Unidirectional Ca uptake rates in control larvae over time loosely corresponded with whole body Ca accumulation over time. However, there was a latent period in between the increase in Ca uptake rate and the increase in whole body Ca content as the ion presumably required time to accumulate. A low initial Ca uptake rate at 12 h (Figure 2-1) corresponded with constant low levels of Ca in the larvae for the first 48

h of development (Figure 2-3a). The increase in Ca uptake at 48 h (Figure 2-1) corresponded with an increase in Ca content at 60 and 72 h (Figure 2-3a). Also, the plateau in Ca uptake rates from 60 to 84 h of development (Figure 2.1) resulted in a plateau of Ca levels in the larvae between 72 and 96 h (Figure 2-3a). Our results showing Ca accumulation increasing over time are consistent with the idea that Ca is being incorporated into the developing spicule matrix over development (Figure 2-3a).

The Ca flux measurements mentioned earlier are unidirectional and therefore the efflux rate of Ca is unknown. An approximation of the Ca efflux at each stage of development was determined by calculating the theoretical Ca accumulation based on the influx rate of Ca every 12 h and subtracting the measured Ca content of the larvae every 12 h to indicate how much Ca was lost to efflux over development (data not shown). These calculations showed that the majority of the imported Ca was not retained by the larvae over time (96%), however the amount of Ca lost to efflux is lower (64%), after gastrulation. A lower efflux after gastrulation coincides with the potential deposition of ACC onto the spicule to form calcite. Indeed, this may indicate that once the Ca is in the spicule matrix it is less susceptible to being lost through efflux.

Mg, K and Na embryonic accumulation over development

Past work performed has also shown the role of Mg in spicule formation. In vitro experiments by Raz et al. (2003) proved that the presence of Mg in combination with isolated macromolecules from the spicule resulted in the formation of the transient ACC. It can therefore be deduced that Mg and spicule macromolecules play key roles in the formation of ACC as a calcite precursor (Raz et al., 2003). Mg is also structurally important in sea urchin development as $MgCO_3$ is found to be a significant component of the spicule, constituting 5% of its mineral phase (calcite, $CaCO_3$ is the predominant constituent) (Decker and Lennarz, 1988). Indeed an increase in Mg levels after gastrulation (48 h) during generation of the spicule was observed in this research (Figure 2-3d).

Na is another ion of importance during fertilization and early development of the sea urchin embryo. Research by Payan et al. (1981) showed cyclic patterns in Na influx that corresponded to cellular activity and events of early *P. lividius* sea urchin egg division (up until 60 min), such as respiration (Ohnishi and Sugiyama, 1963), and protein synthesis (Mano et al., 1970). Mano et al. (1970) showed that amino acid transport also corresponds with cellular events of early egg division and this transport is known to be Na-dependent (Epel, 1972). Later research by Nishioka and McGwin (1980), confirmed the role of Na in protein synthesis. K has also been implicated in protein synthesis as permeability to K in the early sea urchin embryo has been linked to increased production of protein (Tupper, 1973).

Most of the current literature on Na and K are on the very early stages of egg division (Schuel et al., 1982; Payan et al. 1981; Tupper, 1973) with little focus on the variation in K and Na influx over later development of the embryo. K and Na measurements in our research however were measured all throughout embryonic development and the two ions displayed similar patterns in their accumulation (Figure 2-

3b and c). It would be of interest to perform further studies to investigate whether the variation in Na and K content also corresponds with amino acid transport and protein synthesis in later stages of the development of the embryo and whether this might explain the accumulation pattern of these ions we measured over development.

Conclusion

From our various experiments we conclude that Ca uptake, storage and utilization in the developing sea urchin embryo is highly variable and therefore can be capitalized upon as a potentially sensitive endpoint in toxicity testing. In particular the gastrulation stage of development showed the greatest increase in Ca uptake and accumulation, as it is a key stage in skeletogenesis. This would suggest that it could be a particular sensitive stage of development in which to study the effects of toxicants, particularly Ca disruptors such as metals. This goal is addressed in Chapters 3 and 4.

The findings of this work will hopefully aid in developing sensitive endpoints in order to refine current toxicity tests utilized in environmental quality testing and the development of water quality guidelines.

F**igure 2-1** Ca uptake rates every 12 hrs over the first 96 hours of larval development. Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Values are means ± SEM (N = 6).

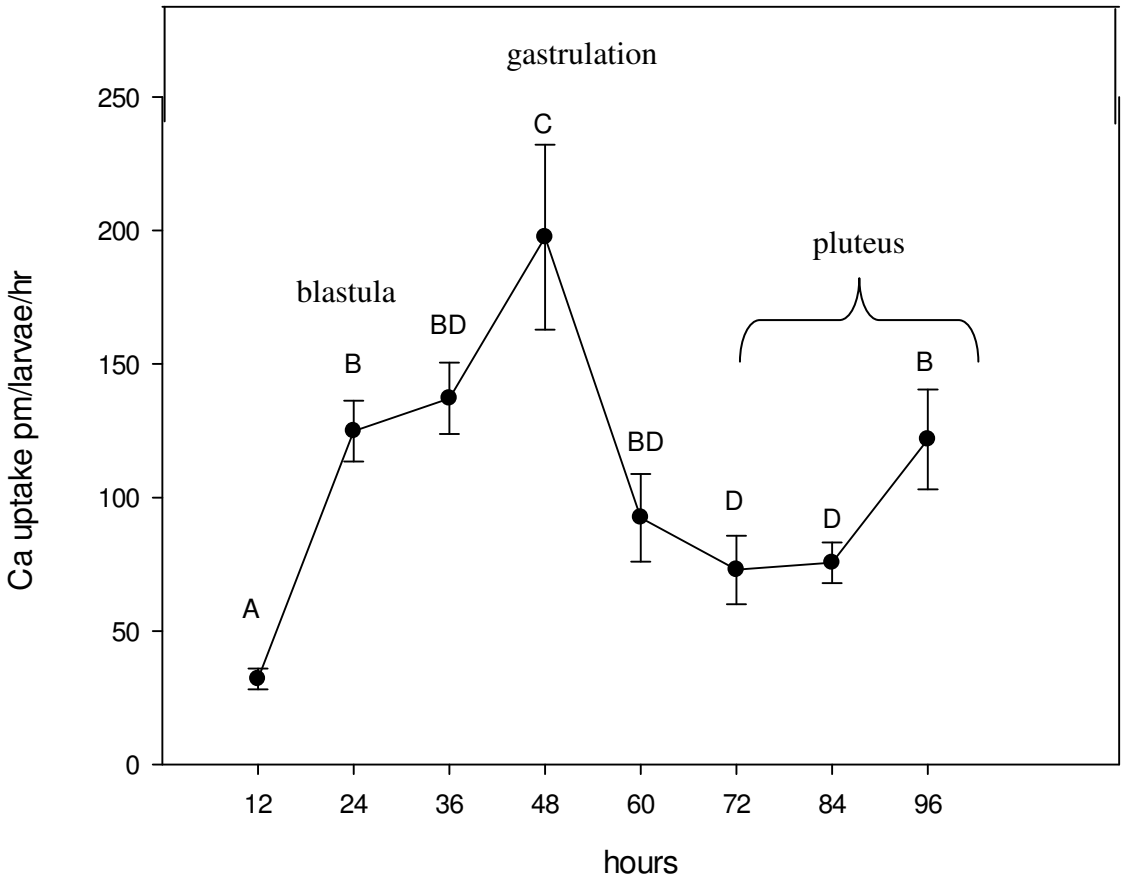

gastrulation
blastula
pluteus
Ca uptake pm/larvae/hr
hours
0
50
100
150
200
250
12
24
36
48
60
72
84
96
A
B
BD
C
BD
D
D
B

Figure 2-2 Larval weights every 12 hrs over the first 96 hours larval development. Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Values are means ± SEM (N = 5).

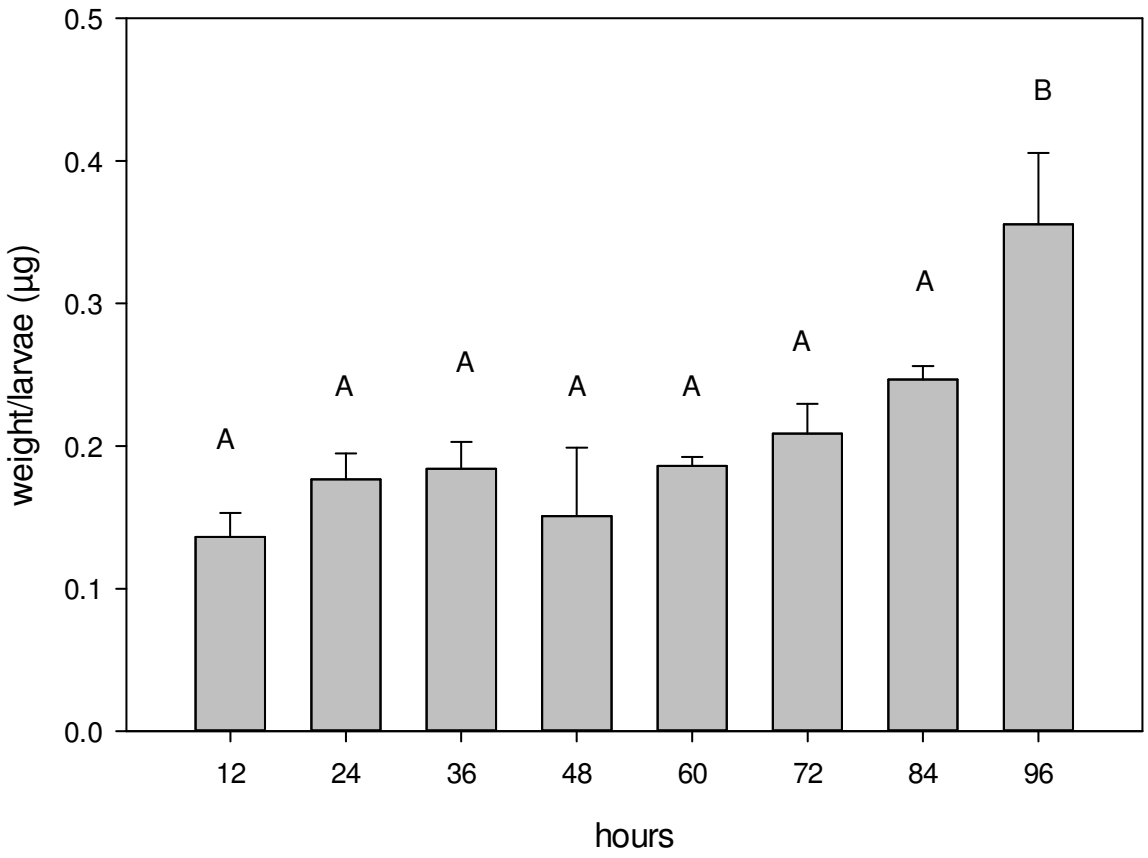
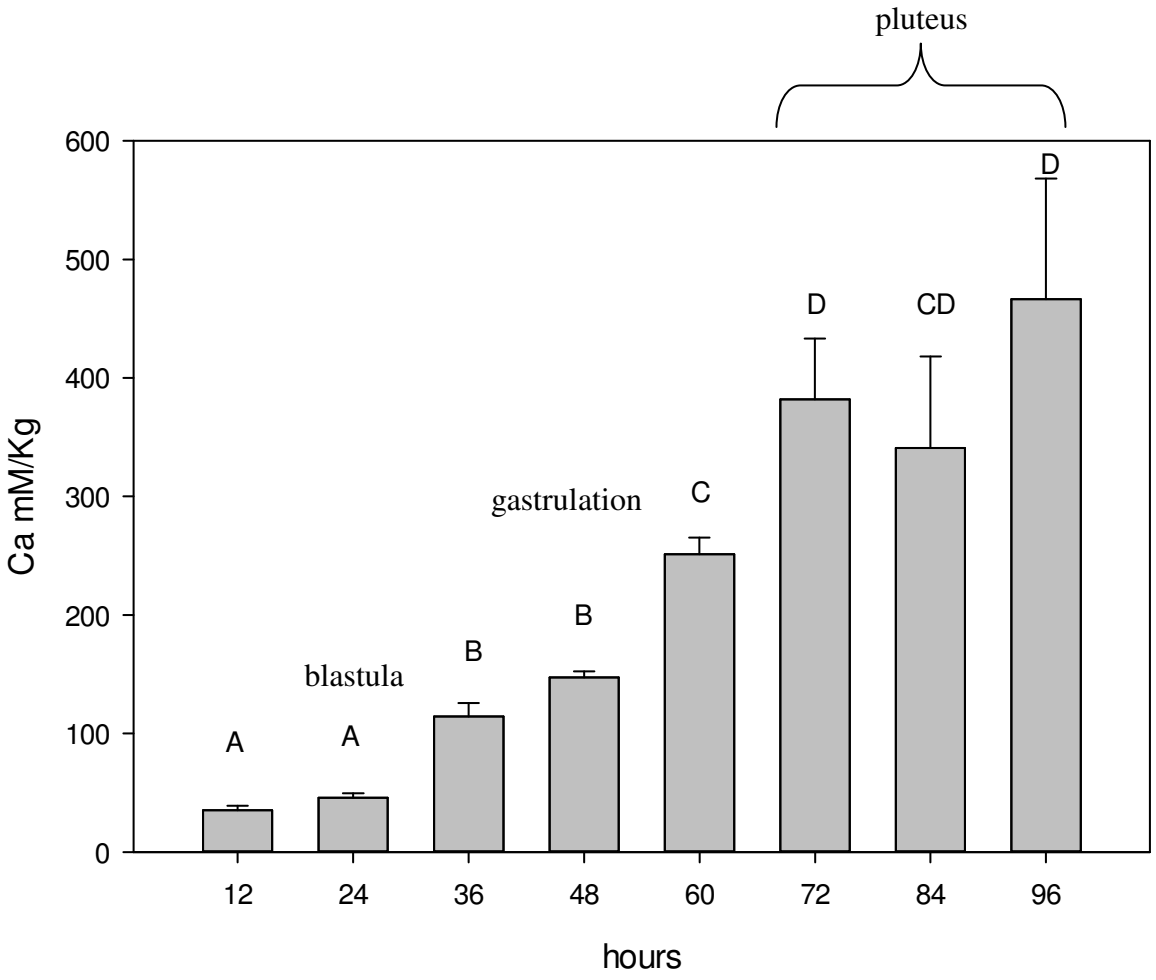
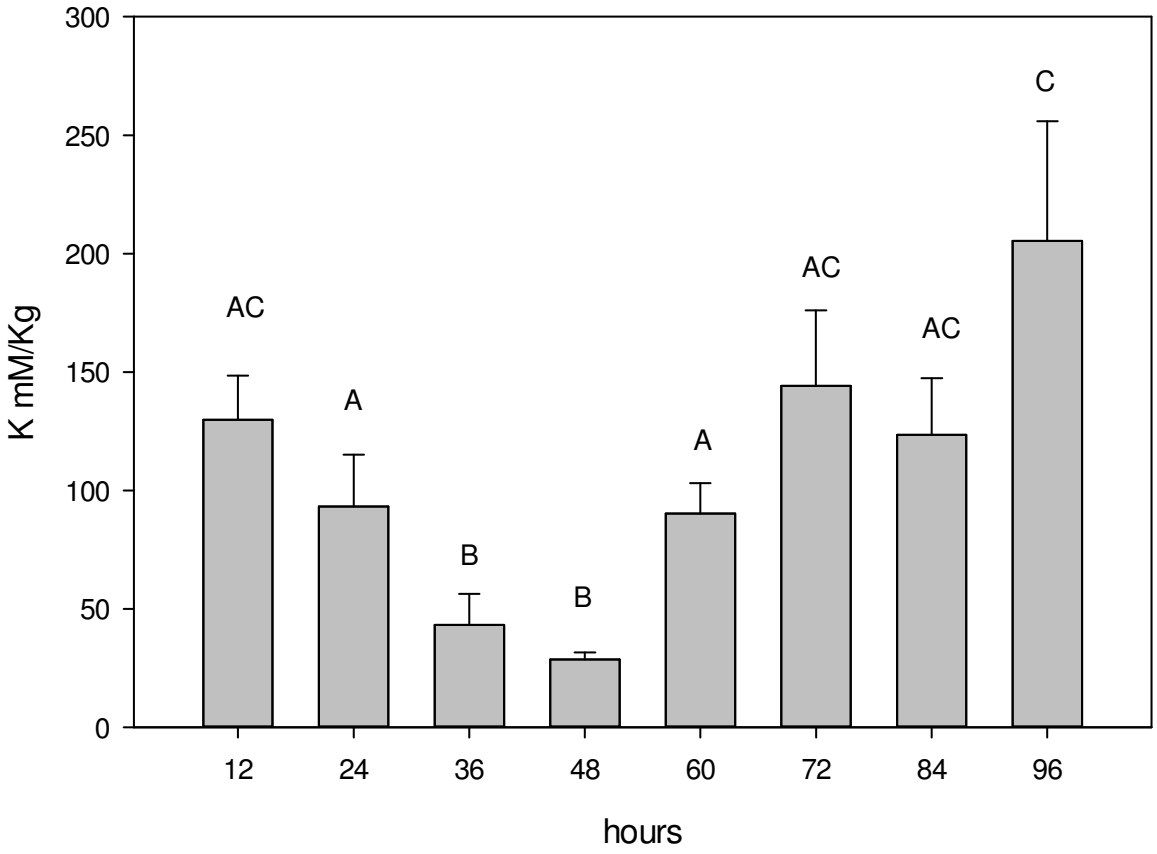

0.5
0.4
0.3
0.2
0.1
0.0
weight/larvae (µg)
A
A
A
A
A
A
A
B
12
24
36
48
60
72
84
96
hours

Figure 2-3 Whole body ion levels in control larvae over 96 hours of development **a)** Calcium **b)** Potassium **c)** Sodium **d)** Magnesium**.** Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Values are means ± SEM (N = 5).

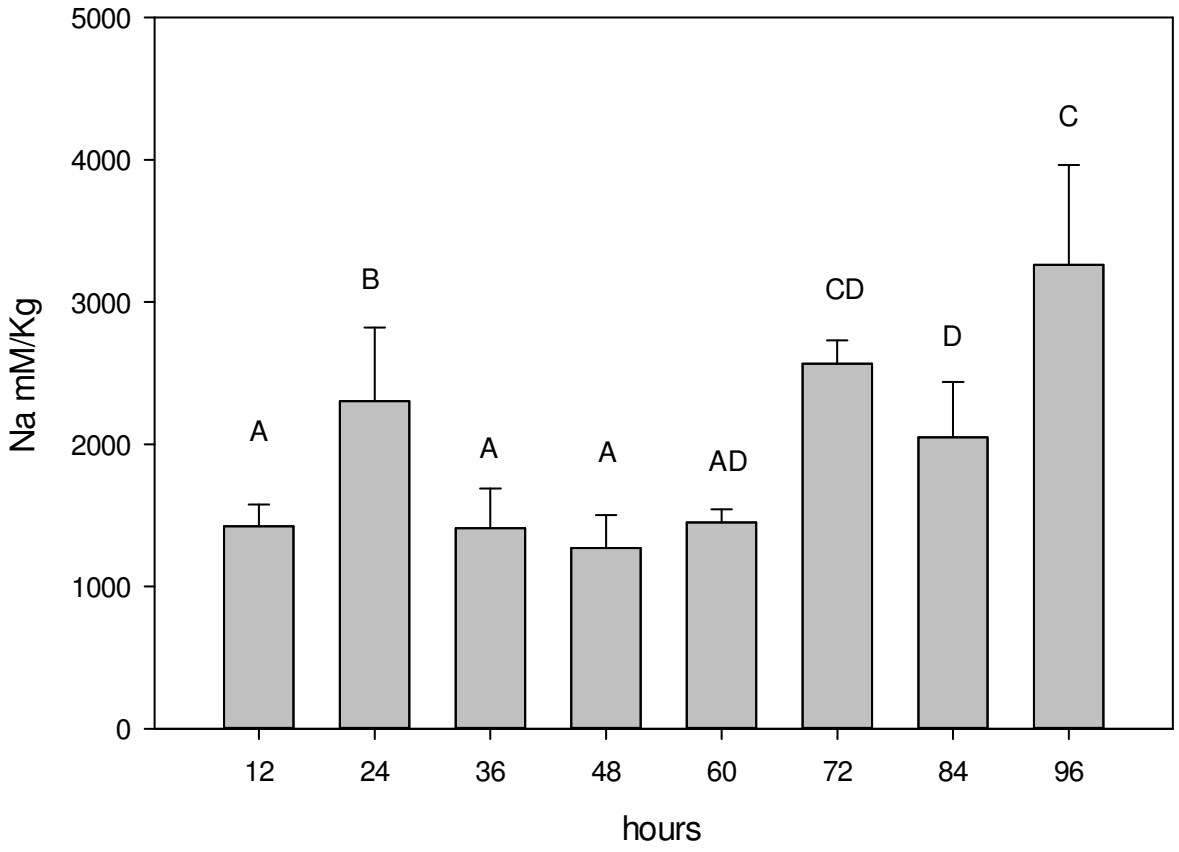
a


pluteus
600
500
400
300
200
100
0
Ca mM/Kg
blastula
gastrulation
A
A
B
B
C
D
CD
D
12
24
36
48
60
72
84
96
hours

b


300
250
200
150
100
50
0
K mM/Kg
AC
A
B
B
A
AC
AC
C
12
24
36
48
60
72
84
96
hours

c


5000
4000
3000
2000
1000
0
Na mM/Kg
A
B
A
A
AD
CD
D
C
12
24
36
48
60
72
84
96
hours

d

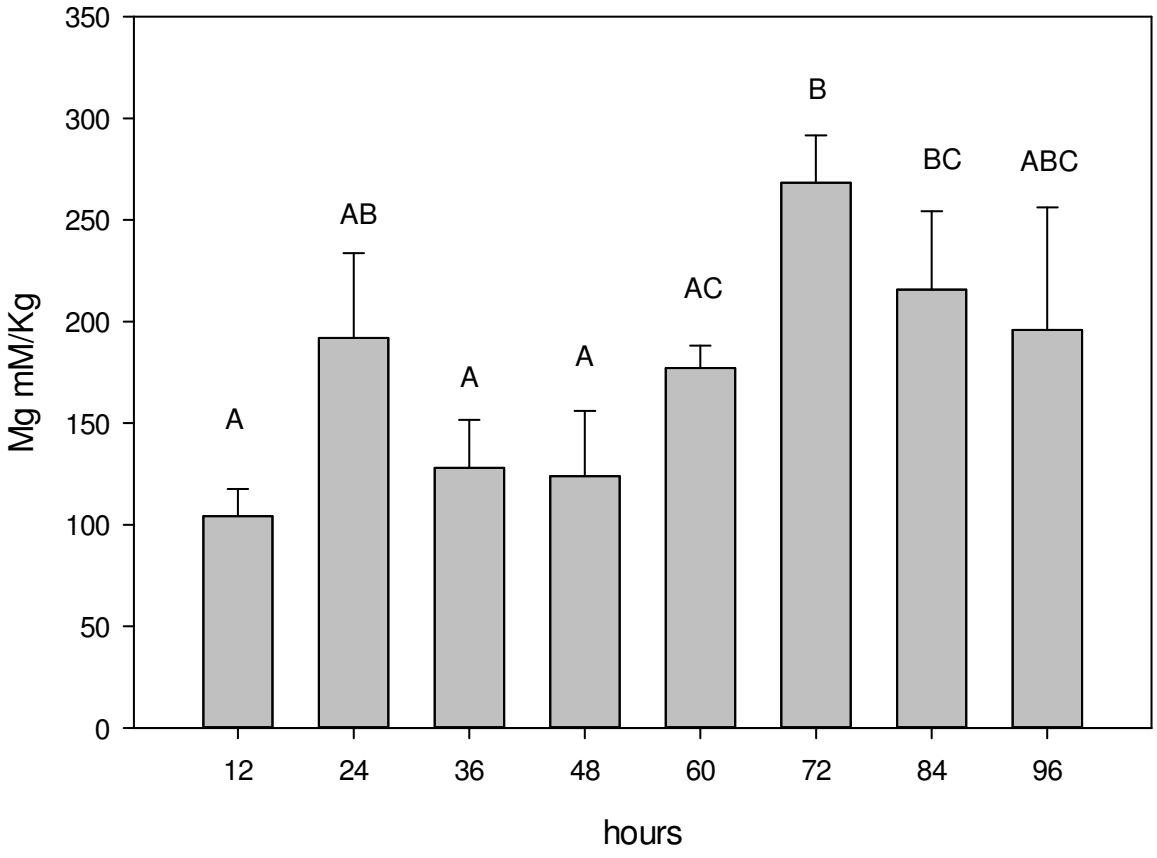
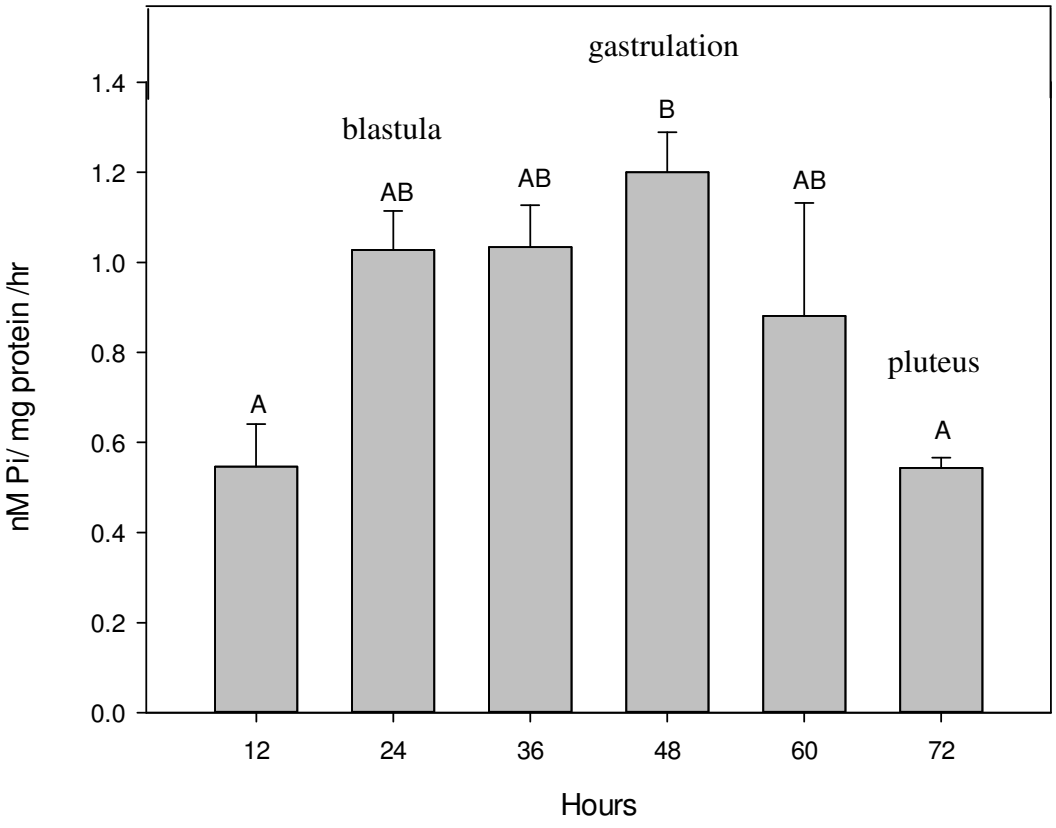

350
300
250
200
150
100
50
0
Mg mM/Kg
A
AB
A
A
AC
B
BC
ABC
12
24
36
48
60
72
84
96
hours

Figure 2-4. Ca ATPase activity over 72 h of development**.** Measures of ATPase activity are expressed as the amount of inorganic phosphate liberated per mg protein per h**.** Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Values are means ± SEM (N = 4).


gastrulation
blastula
pluteus
A
AB
AB
B
AB
A
nM Pi/ mg protein /hr
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
12
24
36
48
60
72
Hours

CHAPTER 3: THE EFFECT OF PB AND ZN ON THE EMBRYONIC AND LARVAL STAGES OF DEVELOPMENT

Abstract

A variety of biological endpoints were utilized in this study to examine the effects of lead (Pb) and zinc (Zn) on the embryonic and larval stages of the purple sea urchin (*S. purpuratus*). Toxicity tests were performed to determine median toxicity threshold values (i.e. EC50: median effective concentration and LA50: median effect accumulation) for Zn and Pb. Growth, unidirectional Ca uptake rates, whole body ion concentrations (Na, K, Ca, Mg), and Ca ATPase activity were also monitored every 12 h over the first 96 h of early development to investigate the mechanisms of toxicity of Pb and Zn. Sea urchin embryos were sensitive to Zn with an EC50 of 2.3 µmol/L (95% C.I. = 1.97–2.71) µmol/L and LA50 of 4.8 (2.16-11.33) µmol/g. Embryos displayed even higher sensitivity to Pb with an EC50 of 0.36 (0.25-0.49) µmol/kg and LA50 of 1.92 (1.67-2.78) µmol/kg. The toxic effect of these metals was increased in combination with DOC. In chronic exposures at 60 µg Pb/L and 139 µg Zn/L both Pb and Zn rendered their toxic effects through disruption of Ca homeostasis. Ca uptake rates were significantly inhibited at various time points over development, in larvae in both metal exposures. This resulted in significantly lower levels of Ca accumulated in the larvae. Interestingly, larvae showed some capacity for recovery as Ca uptake rates and Ca levels returned to control values periodically over development. Metal effects on the whole body levels of three other ions (Na, K, Mg) were modest. Surprisingly, acute exposure to much higher levels of these same metals at various stages during development had negligible inhibitory effects on unidirectional Ca uptake rate, suggesting a mechanism other than direct competition for the Ca uptake sites. We propose studying the toxicity of contaminants periodically over development as an effective way to detect sub-lethal effects, which may not be displayed at the traditional endpoint of 72 h.

Introduction

Across the globe, metals enter marine ecosystems through various anthropogenic and natural modes. At high concentrations metals, including Pb and Zn, are known to be extremely toxic to aquatic organisms, reducing their general survival as well as hampering larval development.

As a xenobiotic, Pb has no known role in the biological processes of organisms and prolonged exposure results in purely toxic effects. It is thought that Pb toxicity stems from its capacity to replace divalent ions such as Zn and Fe, as well as its ability to mimic Ca (Mager, 2012). Not surprisingly, Pb is found in the calcified skeleton of fish exposed

to the metal (Mager, 2012). More study on the mechanisms of Pb toxicity is vital as it is classified as a priority contaminant in European Union regulations on water policy (European Commission, 2001).

Zn is an essential ion as well as a biological stressor, and therefore has considerable importance in the aquatic environment (Glover and Hogstrand, 2002). It is a vital component of over 300 enzymes and other proteins (Vallee and Falchuck, 1993). However at high concentrations, Zn has detrimental effects on the development and survival of aquatic organisms (Eisler, 1993). Similar to Pb, Zn also disrupts Ca homeostasis in freshwater fish through the induction of hypocalcaemia as well as a disturbance of acid-base balance (Spry and Wood, 1985).

While extensive research exists on the effects of Pb and Zn in freshwater environments (Wang, 1987 and Naimo, 1995), there is limited research on the effects of these metals in marine and estuarine environments (Supanopas et al., 2005; França et al., 2005; Kobayashi and Okamura, 2004; Radenac et al., 2001). There are even fewer studies examining the synergistic effects of environmental modifiers such as salinity and dissolved organic carbon (DOC) on the toxicity of Pb (Sánchez-Marín et al., 2007a; Sánchez-Marín et al., 2007b), and to our knowledge, none on Zn in marine organisms.

High levels of DOC are generally protective as the DOC molecules form strong inert complexes with metals, thus reducing their bioavailability (Sanchez-Marin et al., 2007). They vary in protection depending on their sources and fulvic acid (FA) relative to humic acid (HA) content. HAs contain carboxylic and phenolic groups which are known to bind to metals such as Cu and Pb (Sanchez-Marin, 2007). Although DOC is quite protective against metal toxicity to freshwater fish (Grosell et al., 2006; Bringolf et al., 2006; Mager et al., 2010) there is still much uncertainty about the effects of these organic molecules on Pb and Zn toxicity in marine environments. Therefore, more research needs to be performed in order to reduce the uncertainty surrounding marine water quality guidelines. This is of particular importance here in Canada where the authorities (with the exception of Pb in British Columbia) have yet to establish marine water quality criteria for Pb and Zn (Mager, 2012; Hogstrand, 2012).

Another important factor in reducing uncertainty surrounding environmental regulations is to account for the influence of assimilation and metabolism of contaminants by aquatic organisms. Sea urchin embryonic bioassays in particular have been historically utilized as a means of monitoring water quality (Kobayashi, 1971). For the purposes of the current research, the rapidly developing sea urchin is an ideal test organism as it allows us to study the effects of these metals on different cellular processes as they appear in the developing embryo. This is an effective way of pinpointing mechanisms of toxicity. Mechanisms of toxicity, such as sub-lethal effects on molecular and biochemical processes, are important factors to consider when developing policy. They shed light on more sensitive endpoints, which can be detected earlier and at lower concentrations than traditional endpoints such as mortality and inhibited growth.

The objective of the current research was to elucidate the mechanisms of toxicity of Pb and Zn periodically over the first 96 hours of development of the purple sea urchin (*S. purpuratus*). Our research focused specifically on Ca homeostasis as it was

hypothesized that it would be a target of metal toxicity considering the antagonistic relationship of metals with Ca (Rogers et al., 2004; Spry and Wood, 1985). To test this hypothesis, a variety of endpoints of Ca homeostasis were analyzed including unidirectional Ca uptake in acute and chronic Pb and Zn exposures and Ca accumulation and Ca ATPase activity in chronic Pb and Zn exposures. Regulation of Na, K, and Mg concentrations was also monitored in Pb and Zn exposed larvae. Through our research we hope to learn more about the different effects of the metals on each stage of early development.

Materials and methods

Experimental Organisms

Reproductively ripe adult sea urchins (*S. purpuratus)* were obtained by scientific diving teams, from the natural benthic populations of Barkley Sound, B.C., Canada (48°50'30" N, 125°08'00" W). The sea urchins were held in aerated tanks supplied with flow-through seawater (32 ppt) at 15 ˚C to keep gonads ripe and prevent premature spawning or reabsorption of gametes. Only minimal handling of the urchins was permitted so as not to inflict unnecessary stress on the brood stock.

Embryo exposures

Spawning was induced through an injection of 1 ml of a 0.5 M KCl solution into the haemocoel of the adult sea urchin following a method described by Hinegardner (1975). Spawning females were placed upside down on 50 ml Falcon™ tubes containing filtered (0.2 µm Steritop™ filter– Millipore, Billerica, MA, USA) seawater (32 ppt), into which the eggs were collected. The eggs from different females were then pooled by filtration through a mesh into a single beaker. Filtration removed any debris or detritus from the egg solution. A drop of sperm from the spawning males was diluted in 50 ml of filtered (0.2 µm) seawater. This diluted sperm (1 ml) was then added to the pooled sea urchin eggs to initiate fertilization. The solution was gently stirred periodically to facilitate fertilization, which was normally achieved in under 0.5 h. Fertilization success was determined under a microscope, through the appearance of a fertilization membrane around each egg. Once fertilization of 80% of the eggs was achieved, the egg solution was agitated gently to ensure homogeneity of eggs in suspension and the density of eggs in 1 ml of the egg solution was determined under the microscope. The egg solution was diluted using filtered seawater until the desired concentration of eggs in the stock solution was obtained. The egg stock solution was required to be at a density which would result in a final concentration of 1200 eggs/L exposure solution. After the embryos were added to each replicate of exposure solution they were then allowed to develop in an incubator at 15 °C with 14 h light: 8 h dark light cycle for the desired time of each test.

Salinity series

A salinity series was prepared to determine the salinity threshold for normal

development of *S. purpuratus* larvae. Full strength seawater was diluted using nanopure water to achieve a series of salinities (100%, 90%, 80%, 70% and 60%). The salinity test was conducted in 15-ml polyethylene vials with 5 replicates per salinity. Fertilized eggs were then added to each replicate within 4 hours of fertilization. The test solutions were covered and incubated at 15 °C for 72 h. After 72 h the percentage normal vs. abnormal embryos were determined by counting 100 embryos in each replicate under the microscope. From this data, the salinity threshold for the successful development of sea urchin embryos was determined. Based on these results, all further tests were performed with 100 % sea water (32 ppt).

Metal Exposure Solutions

Pb and Zn metal stock solutions were made by diluting their respective inorganic salts ($Pb(NO_3)_2$ and $ZnSO_4$), (Sigma-Aldrich; St. Louis, MO, USA - trace metal grade) in deionized water. These stock solutions were stored in Nalgene™ bottles (Nalgene™ Rochester, NY, USA) under refrigeration. Metal exposure solutions were made by adding the required volumes of metal stock solutions to filtered 100% seawater (0.2 µm) to obtain the desired metal concentrations. Exposure solutions were prepared 24 h in advance of the toxicity tests to allow time for equilibration of the metal salts with seawater in the test containers.

Exposures for the developmental work were set up to ensure that 5 replicates of each exposure (control, Pb and Zn) could be sampled every 12 h over 96 h of development in order for various endpoints to be analyzed.

Zn and Pb EC50 toxicity tests

These tests were performed in 15-ml polyethylene vials with 5 replicates per concentration. Six concentrations of Zn (control, 3.2, 10, 32, 100, 320 and 1000 µg/L, nominal values) were tested to determine median toxicity threshold levels for water Zn median effective concentration (EC50) and whole body Zn burden (LA50). Parallel tests for Pb toxicity were performed by S. R. Nadella and M. S. Tellis, and are reported here for comparison. The concentrations of Pb tested were (control, 3.2, 10, 32, 100, 320 and 1000 µg/L, nominal values)

Fertilized eggs were added to the exposure solutions within 4 hours of fertilization to achieve a final concentration of 1200 eggs/L of exposure solution. The resulting embryos in the exposure solutions were covered and allowed to develop in an incubator at 15 °C with 14 h light: 8 h dark light cycle for 72 h.

All toxicological threshold bioassays were standard ASTM and US.EPA development tests with the proportion abnormal versus normal (i.e. morphologically normal pluteus larvae) at the end of 72 h scored as the endpoint (ASTM 1994; US EPA 2006). At the 72 h mark, 100 embryos in each replicate were counted under a microscope and the number of abnormal versus normal were scored. Tests were only deemed valid if a minimum of 80% of the larvae in each control replicate were normal (ie. had reached the pluteus larval stage) by 72 h. The EC50 values for Zn and Pb were calculated by

ToxCalc v5.0.32 (Tidepool Scientific Software, McKinleyville, USA), as outlined below in the section on statistics and calculations.

DOC and Zn toxicity tests

To test the potential protective effects of DOC against Zn toxicity, the same Zn EC50 toxicity test described previously was performed again, this time with the addition of either Nordic Reservoir DOC (purchased as freeze dried powder from the International Humic Substances Society, St. Paul, MN, USA) or Inshore DOC (collected by the laboratory of Dr. Adalto Bianchini off the coast of Rio Grande do Sul, Brazil). The Inshore DOC had been extracted through solid phase extraction by a method described by Rodrigues and Bianchini (2007). This method utilizes Bond Elut PPL (a styrenedivinylbenzene polymer – Varian Inc., Palo Alto, CA, USA) to extract the DOC from the water.

A series of Zn solutions (0, 3.2, 10, 32, 100, 320 and 1000 µg/L, nominal concentrations) were prepared and spiked with either type of DOC to achieve the desired DOC test concentrations (3 mg/L and 12 mg/L, nominal concentrations). Solutions were mixed thoroughly using a magnetic stir bar and were left overnight in the dark to equilibrate. A concentrated volume of fertilized eggs (less than 4 h old), were added to the test solution the following day to achieve a final concentration of 1200 eggs/L. The exposure solutions were covered and incubated at 15 °C for 72 h. At the 72 h mark, 100 embryos in each replicate were counted under a microscope and the percentage of abnormal versus normal larvae were recorded. Test validity criteria and EC50 calculations were applied as described in the previous section.

Zn and Pb LA50 toxicity tests

In order to determine the body burden of Zn corresponding to 50% lethality (LA50), a range of Zn exposure solutions (0, 3.2, 10, 32, 100, 320 and 1000 µg/L, nominal concentrations) were prepared with 5 five replicates of 500 ml each, for every concentration of Zn. Parallel tests to determine the LA50 for Pb were performed by S.R. Nadella and M. S. Tellis , and are reported here for comparison. The concentrations of Pb tested were (0, 3.2, 10, 32, 100, 320 and 1000 µg/L, nominal concentrations). These solutions were left to equilibrate overnight. Fertilized eggs were then added to each exposure solution so that the final concentration was 1200 eggs/L exposure solution. At the end of 72 h of exposure each replicate was filtered by vacuum pump through a 0.45 µm filter (Nalgene^{TM} Rochester, NY, USA) and resulting larvae collected on the filter paper were rinsed with nanopure water. The filter paper with the collected larvae from each replicate was then placed in an Eppendorf^{TM} tube and left to dry at room temperature. After the filter paper was dry, 1 ml of full strength HNO_3 was added to each tube to digest the sample. Metal accumulation was then measured in the digested samples at each exposure concentration. Using flame atomic absorption spectroscopy (220FS; Varian Instruments, Palo Alto, CA, USA) for Zn measurements and graphite furnace atomic absorption spectroscopy (220, Varian Instruments, Palo Alto, CA, USA) for Pb measurements. Reference standards used were TM24 and TM25 (Environment Canada

certified reference material, recovery 90 % - 95 %).

The LA50 was then calculated from the regression of log of metal accumulation against the logit of percent abnormality, as outlined below in the section on statistics and calculations.

Whole body ion concentrations (Ca, Mg, Na and K) after 72 h of metal exposure were also measured in the LA50 samples using flame atomic absorption spectroscopy (220FS; Varian) to determine the relationship between metal exposure and accumulation and whole body ion content.

Radioactive ^{45}Ca uptake rate measurements in developing larvae under either acute or chronic exposure to Pb or Zn.

Unidirectional uptake rates of Ca from the water, as determined by ^{45}Ca incorporation, were measured in separate batches of embryos at 12-h intervals over the first 96 h of development. As described below, tests were performed with larvae either acutely or chronically exposed to Pb or Zn.

For each flux rate determination, embryos were sampled from each replicate by gently stirring it to ensure homogeneity of embryos in suspension. Then a small volume was removed from each of the 6 replicates. The extracted volume was decreased to a few ml by filtering out some of the seawater by gravity using a Steritop^{TM} filter (0.2 µm – Millipore, Billerica, MA, USA) leaving a concentrated embryo solution. This concentrated embryo solution was re-suspended with fresh seawater and reduced in volume again. This was repeated 3 times to wash the embryos. The embryos were finally resuspended in fresh seawater to achieve a nominal target density of 2500 embryos/ml for the ^{45}Ca uptake rate measurements.

Ca flux rate measurements were performed by incubating 0.5 ml of sea urchin embryos suspension with 0.5 ml of radioactive ^{45}Ca (0.17 µCi/ml as $CaCl_2$, PerkinElmer, Woodbridge, ON, Canada) in seawater in 2-ml Eppendorf^{TM} tubes for 20 min. The flux period was based on preliminary time series experiments in which 20 min was determined to be optimal for ^{45}Ca uptake analysis (see Chapter 2).

On completion of the 20-min flux period, the larvae were removed from the Eppendorf^{TM} tube via a 1 ml syringe. A 45-µm syringe tip filter (Nalgene^{TM} Rochester, NY, USA) was then attached to the end of the syringe and the flux solution held in the syringe was injected through it, leaving the embryos on the filter. Then 10 ml of fresh seawater was immediately passed through the filter to wash the larvae. The filter was then reversed and 3 x 1 ml (i.e. each ml separately) of fresh seawater was passed through the filter and collected in a scintillation vial to recover the embryos. Scintillation fluid (5 ml; Aqueous Counting Scintillant, Amersham, Little Chalfont, UK) was added to each vial and the ^{45}Ca in the sample was measured using a beta counter (PerkinElmer, liquid scintillation analyzer, tri-carb 2900TR, Woodbridge, Canada). A dummy run (without ^{45}Ca) of the flux procedure was performed at each time point and the embryos recovered from the filter were counted under the microscope to determine embryo concentrations used in the flux measurements.

Unidirectional Ca uptake rates per larva were calculated from the counts per

minute of each replicate (CPM), mean specific activity (SA), number of larvae in each replicate and experimental time (t) and expressed as pmol Ca/larvae/h:

Ca uptake = (CPM/SA) * (1/# of larvae)*(1/t)

Note: Specific activity was calculated by dividing the Ca (pmol/ml) in seawater by ^{45}Ca radioactivity (CPM/ml) .

A spike method was used to determine the effect of an acute exposure to Pb or Zn on unidirectional Ca uptake rates in larvae at various times of development. A 100 mg/L Pb or Zn stock solution was used to spike each flux vial to obtain the desired final concentration of metal in the flux medium. The 20-min flux period simultaneously served as an acute metal exposure to the larvae. Three concentrations (6 replicates each) of each metal were tested at each time point. The concentrations chosen were approximately the EC50, 2x the EC50 and 4x the EC50 values for each metal. Therefore the actual nominal concentrations tested were 75, 150 and 300 µg/L for Pb and 150, 300 and 600 µg/L for Zn. Although these high metal concentrations would most certainly result in larval mortality in a chronic exposure, the 20 min acute exposure period would not be a long enough exposure for these metal levels to cause damage to the larvae. These concentrations were chosen purely for the study of competitive effects of metals on Ca uptake.

For the chronic exposures. Pb and Zn exposure solutions (six replicates each) of a single concentration (approximately 30 % below their respective EC50s) were prepared in 500 ml plastic tubs. Therefore the actual nominal concentrations tested were 54 µg/L (Pb) and 106 µg/L (Zn) respectively. Exposure solutions were prepared following the same protocol for test solution preparation as the EC50 toxicity tests. The fertilized eggs were added to the solution and were sampled for unidirectional Ca uptake rate measurements every 12 hours over the first 96 hours of development.

Ca ATPase enzyme analysis

At each 12-h time point, 5 replicates of each chronic exposure (control, Pb and Zn) were sampled. The volume of each replicate was reduced to 20 ml through filtering the solutions by gravity through a 0.45 µm filter (Nalgene™ Rochester, NY, USA). The concentrated embryos were resuspended in fresh seawater and concentrated again. This was performed 3 times to wash the larvae. The washed, concentrated larvae were then centrifuged at 12000 g for 5 min, the supernatant was decanted and the resulting pellet was transferred to an Eppendorf™ tube and frozen at -70 °C for later analysis.

Ca^{2+}ATPase analysis was performed through a method which measured liberation of inorganic phosphate by the ATPase enzyme (Vijayavel et al., 2007).
Ca^{2+}ATPase activity was determined as the amount of inorganic phosphate per mg of protein per hour released in a medium containing 80 mM NaCl, 5 mM $MgCl_2$, 3 mM ATP, 20 mM Tris–HCl (pH 7.4), 0.5 mM $CaCl_2$ and 1 mM ouabain. Activities were normalized to the protein content of the homogenate, as determined using BSA standards (Sigma-Aldrich) and Bradford's reagent (Sigma-Aldrich; Bradford, 1976).

Ion and metal accumulation analysis and larval weight measurements

Every 12 hours over the first 96 hours of development, 5 replicates of each chronic exposure treatment (control, Pb and Zn) were sampled. The embryos in each replicate were filtered via a vacuum pump through a 0.45 µm filter (Nalgene™ Rochester, NY, USA) and rinsed with nanopure water. The filter paper was then placed in an open Eppendorf™ tube and left to dry at room temperature. Ion levels (Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}) were then measured using flame atomic absorption spectroscopy (220FS; Varian Instruments, Palo Alto, CA, USA) and metals were measured using graphite furnace atomic absorption spectroscopy (220, Varian Instruments, Palo Alto, CA, USA). Reference standards used were TM24 and TM25 (Environment Canada certified reference material, recovery 90 % - 95 %).

The dried filter with collected embryos on it was weighed. This weight minus the filter weight divided by the number of larvae collected was taken to be the mean dry weight of the developing embryo or larvae.

Metals and Ca analysis in seawater

Metal concentrations of seawater exposure solutions in the tests were determined using a method developed by Toyota et. al. (1982) in which the metal is precipitated from solution in order for it to be analyzed without interference from the many electrolytes in saltwater. To do this, 1 µl of lanthanum oxide (10 mg La/ml) and 7.5 µL of 1 M Na_2CO_3 were added per ml of sample, which was kept in a water bath at 80^0C for 30 min. The flocculate solution was then centrifuged at 3000 g for 15 min, the supernatant was decanted and discarded, and the pellet was then dissolved in 1ml of 1 N HNO_3. Metal concentrations in the acid solution were then measured in the standard manner using flame atomic absorption spectroscopy (220FS; Varian, Varian Palo Alto, CA, USA) for Zn and graphite furnace atomic absorption spectroscopy (220, Varian) for Pb. Reference standards used were TM24 and TM25 (Environment Canada certified reference material, recovery was between 90 - 95 %).

Ca levels were also measured in seawater against commercial standards (Fisher Scientific, Toronto, ON, Canada) by flame atomic absorption spectroscopy (220FS; Varian Instruments, Palo Alto, CA, USA). This was to provide seawater Ca concentrations for the specific activity calculations mentioned earlier.

DOC analysis

Seawater samples were first passed through a 0.45 µm filter and then measured for total carbon (TC) and inorganic carbon (IC) using a Shimadzu TOC analyzer (5050A, Mandel Scientific, Canada). IC and TC standards were prepared according to Shimadzu protocol. DOC was calculated to be the difference between IC and TC.

Statistical analysis and calculations

Statistical analysis was performed with SigmaPlot 10.1. Student's t-tests (two-tailed) were used to determine differences between control and metal-exposed larvae at

specific times. One-Way ANOVAs were used to detect variation among multiple treatment groups and where the F value indicated significance, Fishers LSD test post hoc tests were used to identify specific significant differences. Prior to the test, all data were checked for homogeneity of variances and normality of distribution, and where necessary were transformed using natural logarithm or square root functions. All data are presented as means ± SEM (N, number of replicates) on non-transformed data. Differences were considered significant at $p < 0.05$.

An Environmental Toxicity Data Analysis Software Tox Calc™ package (Tidepool Scientific Software) was utilized to calculate the EC50 values with 95% confidence intervals (CI). The responses (percentage abnormality) and measured metal concentrations of each test concentration were used as inputs to the program. Responses were considered significantly different when their 95% CI did not overlap (Environment Canada, 2005).

The LA50 values were calculated by linear regression of the logit transformation of abnormality (ln(a/(1-a)), where a represents percentage abnormality) against the log of metal burden. The LA50 was the metal burden at which logit of abnormality equalled zero. This statistical linear interpolation technique also gives you the 95% confidence intervals.

Results

Salinity threshold

Sea urchin embryos showed extreme sensitivity to reductions in salinity with less than 80% survival at 90% seawater and no survival beyond 70% seawater (Figure 3-1). Therefore all further tests were performed in 100% seawater.

Median Zn and Pb toxicity thresholds

Larvae showed high sensitivity to Zn with an EC50 of 2.3µmol/L (129-177) (Figure 3-2a). The LA50 for Zn was calculated to be 4.8 (2.16-11.33) µmol/kg (Figure 3-2b).

In parallel experiments on Pb toxicity by S.R. Nadella and M. S. Tellis, the EC50 [0.36 (0.25-0.49) µmol/L] was significantly lower than that for Zn as was the LA50 [1.92 (1.67-2.78) µmol/kg] (data not shown).

Influence of DOC on Zn toxicity

Surprisingly, DOC in itself proved to be toxic to the embryos, as there was 12 % and 15 % less survival in controls with 3 mg/L and 12 mg/L of Brazilian inshore DOC respectively, than controls without DOC. Nordic reservoir DOC proved to be more toxic than Brazilian inshore DOC with no survival of embryos even in the absence of Zn (Figure 3.3a and b)

Inshore DOC collected in Brazil in combination with Zn caused a decrease in the Zn EC50 from 151 µg/L to 13.7 µg/L when Zn treatments were spiked with 3 mg/L DOC

(Figure 3-3a). Adding a higher concentration (12mg/L) of the same DOC proved to have higher toxicity to the embryos (EC50 10.8 µg/L) (Figure 3-3b). Addition of Nordic Reservoir DOC (3 mg/L and 12 mg/L) to the exposure medium was even more toxic, resulting in no survival of the embryos in controls or at any concentration of Zn.

Measured versus nominal metal levels during 96 h development exposures

In the first chronic exposure (96 h), larvae were exposed to nominal concentrations of either Pb or Zn targeted to be 30 % below their respective EC50s in order to assess impacts Ca uptake, ions and metal accumulation over 96 h. The same control set was used for both metals. The actual measured Zn concentrations in these tests were 139 µg/L and background levels of Zn in the water were 34 µg/L. The actual measured Pb concentrations were 60 µg/L with background levels of 12 µg/L.

In the second chronic exposure (72 h) in which larvae were sampled for Ca ATPase, targeted nominal levels were the same as the first exposure and measurements indicated that the actual concentrations used were very similar to those in the first series. Zn was measured to be 127 µg/L and Pb levels were measured to be 65 µg/L.

Unidirectional Ca uptake

Acute exposure to a range of Pb and Zn concentrations did not have significant effects on unidirectional Ca uptake, as measured by ^{45}Ca incorporation, at any stage of development (Figures 3-4a and 3-5a).

However, inhibition of ^{45}Ca uptake was observed at certain time points over development when measuring uptake in chronically exposed larvae. In larvae chronically exposed to Pb (52 µg/L), inhibition of ^{45}Ca uptake was recorded at the time points when ^{45}Ca uptake was highest in control larvae, i.e. at the blastula stage (24 h), gastrulation (48 h), and during the pluteus larval stage (84 h and 96 h) (Figure 3-4b). Similar to Pb, chronic exposure to Zn (106 µg/L) resulted in inhibition of ^{45}Ca uptake during the blastula stage (24 h) as well as at gastrulation (48 h). Inhibition of ^{45}Ca uptake was also seen during the early pluteus larval stage (72 and 96 h) (Figure 3-5b).

Ca ATPase activity

Pb exposure (54 µg/L) caused significant increases in whole body Ca ATPase activity during the gastrulation stage at 48 h and at 60 h (Figure 3-6a).

Whole body Ca ATPase activity was significantly lower at 24, 36 and 48 h of development in Zn exposed (106 µg/L) larvae (Figure 3-6b). Levels of Ca ATPase however were higher at 72 h during the pluteus larval stage.

Larval weights

Control larvae displayed a significant increase in weight at 96 h. Zn exposed larvae displayed a similar increase in weight over time (Figure 3-7b), which was not seen in Pb exposed larvae (Figure 3-7a). Both Pb and Zn exposed larvae were significantly heavier than controls at 60 h.

Whole body ion content at 72 hours of development

In the larvae used for the LA50 tests, whole body ion concentrations of K, Na and Mg at 72 h did not differ over a range of Zn exposure concentrations (Figure 3-8 b, c and d). However, Na and Mg were significantly lower in higher concentrations of Pb exposure (Figure 3-9 c and d). Ca levels were not different from controls in Zn exposed larvae, but were significantly lower in comparison to controls, at high concentrations Pb (Figures 3-8a and 3-9a).

Whole body ion content of larvae over development

A somewhat different picture of ionoregulatory effects was seen in larvae chronically exposed to sublethal levels of Pb and Zn, and sacrificed at 12-h intervals for analysis of whole body ion concentrations.

Most notably, in organisms exposed to Zn (106 µg/L) over development, whole body Ca levels were lower than controls at every time point after 24 hours, with the exception of the 84 hour time point (Figure 3-10a). K levels were generally not affected by this chronic Zn exposure apart from at 24 hours where they were significantly lower with respect to controls (Figure 3-10b). Mg in Zn exposed larvae was significantly higher than controls at 12 and 24 hours of development and was significantly lower than controls at 96 hours of development (Figure 3-10d).

Larvae chronically exposed to Pb suffered lower levels of Ca during the initial stages of development at 24, 36 and 60 hours of development. However Ca levels in Pb exposed larvae returned to control amounts by 72 hours onwards (Figure 3-11a). Chronic Pb exposure (54 µg/L) did not affect K and Na levels over 96 hours of development (Figure 3-11b and c). Exposure to Pb caused an increase in Mg levels at 12 hours of development, but had no effect on Mg over the rest of the 96 hours of development (Figure 3-11d).

Whole body metal accumulation of larvae over development

There was no significant amount of Zn accumulated in Zn (106 µg/L) exposed larvae by the end of the 96 hours exposure period. However, Zn exposed larvae had significantly higher Zn content than controls at the 36 and 72 h time points (Figure3-12a).

Larvae chronically exposed to Pb (54 µg/L) showed significant accumulation at every time point after 12 h, over the 96 h development period. Pb accumulation in Pb exposed larvae also increased significantly over time (Figure 3-12b).

Discussion

Classic toxicity testing showed *S. purpuratus* larvae to be highly sensitive to both Zn and Pb, as well as to changes in salinity. Through further analysis of various biomarkers over development, both Pb and Zn toxicities were determined to be associated with disruption of Ca homeostasis.

Salinity

Sea urchin larvae were shown to be extremely sensitive to changes in salinity with

less than 80% survival at 90% sea water (Figure 3-1). Echinoderm larvae in general are very poor ionoregulators and are viable only within a narrow margin of salinities (Kinne, 1971). Developing urchin embryos are able to maintain ion homeostasis by modifying cell membrane potential, which regulates cell permeability to ions. However ionoregulation only occurs within a narrow range of salinities as passive diffusion of ions into the embryo through transporters and voltage-gated channels is highly influenced by the concentrations of ions in the external medium (Hagiwara and Jaffe, 1979). Salinity was therefore not altered for the remainder of the research.

Pb and Zn toxicity

Sea urchin embryos were very sensitive to Zn with an EC50 of 2.3 µmol/L (95% C.I. = 1.97–2.71 µmol/L) and LA50 of 4.8 (2.16-11.33) µmol/kg. Embryos displayed even higher sensitivity to Pb with an EC50 of 0.36 (0.25-0.49) µmol/L and LA50 of 1.92 (1.67-2.78) µmol/kg. This is consistent with the principles of the BLM in that Pb with a higher log K value of 6 compared to a log K value of 5.3-5.5 for Zn (freshwater values reviewed by Niyogi and Wood, 2004) results in a lower EC50 and LA50 than Zn. Put simply Pb is more toxic than Zn, as less Pb is required to invoke equal toxicity. This toxicity is a reflection of the higher affinity (log K) of Pb for the biotic ligand.

DOC

Initial research performed by Arnold (2005) on the interaction of Cu and DOC provided promising evidence of the protective properties of DOC against metal toxicity. The present research however, shows evidence to the contrary when Zn is combined with DOC. Brazilian inshore DOC was shown to increase the toxicity of Zn to sea urchin embryos, evident from a decrease in the Zn EC50 from 2.31 µmol/L to 0.21 µmol/L when 3 mg/L of the DOC was added. The EC50 was further decreased to 0.16 µmol/L upon addition of a higher concentration (12 mg/L) of the same type of DOC; however this was not a large enough decrease to indicate dose dependent toxicity of the DOC in combination with Zn. Nordic reservoir DOC was more toxic than Brazilian inshore DOC as there was no survival of embryos at any concentration of Zn upon its addition to the Zn exposure. Interestingly, DOC in itself proved to be toxic to the embryos, as there was less survival in controls with Brazilian inshore DOC added, than controls without DOC. Nordic reservoir DOC again proved to be more toxic than Brazilian inshore DOC with no survival of embryos even in the absence of Zn (Figure 3-3a and b).

There has been much speculation as to why DOC is toxic to developing sea urchins (Sánchez-Marín et al., 2007). Possible explanations include the tendency of humic acids to accumulate on cell surfaces, inducing electrophysiological changes in the cell membrane (Sánchez-Marín et al., 2007). DOM binding to cell surfaces can change cell membrane potential thus opening voltage-gated channels, which could result in increased cell permeability to molecules, including metals. In the case of Pb, cellular uptake appears to be through voltage gated Ca channels (Sánchez-Marín et al., 2007). Therefore a change in membrane potential could result in increased uptake of Pb through

these channels (Sánchez-Marín et al., 2007). Indeed, increased uptake of Pb in the presence of humic acids has been observed in past studies (Sánchez-Marín et al., 2007; Sánchez-Marín et al. 2011). A similar explanation could explain the increased toxicity of Zn in the presence of added DOC.

There is some debate in current literature about the source dependant protectiveness of DOC, with some studies arguing that protection from DOC is independent of the source (Arnold et al., 2006; DePalma et al., 2011) and other studies indicating that effects of DOC on toxicity vary depending on source (review by Al-Reasi et al., 2011). Our study shows that the effects of DOC on toxicity varied depending on the type of DOC used, which may be a result of the source of the DOC. Additionally toxicity of the DOC may be attributed to the DOC not being from a source native to the sea urchins. However this is purely speculative and further tests with different types of DOC are needed.

Brazilian inshore DOC was utilized in the tests based on the availability of this DOC in sufficient quantities for experimentation , and because it was from a specifically marine source. Nordic reservoir was utilized in this research because previous studies in the lab by Nadella et al. (2009) showed protective properties of this DOC against metal stress to *M. trossolus* larvae.

As DOC in this study proved to be toxic to developing larvae and survival of urchin larvae was greatly inhibited with even small decreases in salinity (Figure 3-1 and 3-3a and b), these environmental parameters were not manipulated in subsequent toxicity tests performed. The objective of the work shifted to studying toxicity at regular intervals over the first 96 h of development, as opposed to only looking at toxicity at the 72 h time point. The goal was to determine the developmental stages and processes that are most sensitive to the metals.

Ca uptake over 96 hours of development

Ca uptake by the developing sea urchin showed a variable pattern over 96 h development depending on the differing Ca requirements of each developmental stage. There were no direct competitive effects of Pb and Zn on Ca uptake, evident from a lack of Ca uptake inhibition in larvae acutely exposed to a range of metals, including much higher concentrations than used in the chronic tests, at each stage of development (Figure 3-4b and 3-5b).

In larvae chronically exposed to Pb and Zn, Ca uptake inhibition was observed at several time points over 96 h of development (Figure 3-4b and 3-5b). Interestingly, these chronically exposed larvae mainly displayed inhibition of Ca uptake during developmental stages when Ca uptake rates were highest. This was during the blastula (24 h), gastrulation (48 h) and pluteus (84 h and 96 h) stages for Pb exposed larvae (Figure 3-4b) and during the blastula (24 h), gastrulation (48 h) and pluteus (72 h and 84 h) stages for Zn exposed larvae (Figure 3-5b). This could indicate that the Ca channels were compromised and were unable to meet the increased Ca demands during these stages. Voltage gated Ca channels are inhibited by extracellular transition metals such as Zn, Pb,

Cu and Ni via binding of these cations at negative sites close to the gating apparatus. These metal cations slow the opening rate of these channels by making the voltage required for activation more positive (Gilly and Armstong, 1982). This is not a direct competition with Ca. The extent to which these channels are slowed down depends on the type and dose of metal to which the channel is exposed. A higher dose of the metal does not necessarily result in greater inhibition of uptake by the channel, as sometimes greater inhibition of the channel occurs at lower doses of the metal (Büsselberg et al., 1992).

All metals in the chronic exposures caused greatest inhibition of Ca uptake at the gastrulation stage. This stage of development is known to be an especially critical and vulnerable landmark in development. On completion of this developmental stage, three germ layers (ectoderm, mesoderm and endoderm) and a rudimentary gut are formed and skeletogenesis is initiated. Understandably, abnormalities at this phase often result in complications in later development of the skeleton (Yaroslavetseva and Sergeeva, 2002).

One reason why the gastrulation stage might display such sensitivity to metal toxicity is that the maternal reserves of metallothioneins are depleted by this time. Metallothioneins are a group of low molecular weight proteins, which protect organisms by virtue of their high affinity for metals. Their affinity for metals is a result of their cysteine rich content (Warnau et al., 1996). A decrease in available metallothioneins by gastrulation might leave the embryos vulnerable until an increase in de novo synthesis of these proteins is initiated (Warnau et al., 1996). Newly synthesized metallothioneins may also be more effective in their protective role against metals, as they have not been previously exposed to metals as the maternal metallothioneins might have been. Evidence for this is seen in a return to normal of Ca levels in Pb exposed larvae in stages after gastrulation (Figure 3-11a).

In research by Kobayashi (1980), where larvae were introduced to polluted sites at different stages of development, it was revealed that the first stages of cleavage division, the bastula and pluteus stages, were the least vulnerable stages of development from fertilization to formation of the pluteus. Similar to these findings, we observed no inhibition of Ca uptake at 12 hrs when the embryos were undergoing initial cleavage division, at the blastula stage and for the majority of the pluteus phase being tested (Figure 3-4b and 3-5b).

Whole body ion content over a range of Pb and Zn exposures

Pb exposure caused significant ionoregulatory disruption in the larvae, with lower levels of Ca, K, Mg and Na being observed with increasing Pb exposure (Figure 3-9a, b, c and d). Ca, K, Mg and Na levels did not appear to be affected with increased concentrations of Zn exposure (Figure 3-8a, b, c and d); however, when ion levels in Zn exposed larvae were examined over development, ionoregulatory disturbance was observed.

Whole body ion content over development

Ca and Mg were the only ions that displayed marked differences in larvae chronically exposed to Pb (54 µg/L) and Zn (106 µg/L) over development. Both ions are

integral constituents of the spicule (skeleton) (Raz et al., 2003), which makes disruption of their homeostasis by metals more noteworthy.

In both metal exposures, larval Mg levels were higher than controls during the initial stages of development (Figure 3-10d and 3-11d). Pb exposed larvae exhibited increased levels of Mg at 24, 36 and 60 h of development and Zn exposed larvae displayed higher levels of Mg at 12 h. This could be a compensatory mechanism employed by the larvae to counter metal induced disruption of Ca incorporation.

Ca was the only ion out of the four ions measured that increased in accumulation consistently over the 96 h development period (Figure 3.7a and 3.8a; see also Chapter 2). This underscores the importance of measuring Ca uptake, as it is obvious that Ca has a significant role in sea urchin larval development. A disruption of Ca homeostasis therefore significantly hinders normal sea urchin larval development and is an important mechanism of toxicity.

Ca uptake rates in control larvae over time corresponded with Ca accumulation over time. However, there was a latent period in between the increase in Ca uptake rate and the increase in whole body Ca content as the ion presumably required time to accumulate. Similarly, there was a latent period in between inhibition of Ca uptake by metal exposure and lower levels of Ca accumulated in the metal exposed larvae. In Zn exposed larvae, inhibition of Ca uptake at 24 hours (Figure 3-5b) resulted in lower levels of Ca at 40 hours onwards (Figure 3-10a).). Chronic Zn exposure had a major effect on Ca homeostasis with a great decrease in Ca levels in exposed larvae. However, in our experiment performed a year earlier, larvae exposed to a range of Zn concentrations did not display lower levels of Ca at similar exposure concentrations (Figure3-8d). This could be due to year-to-year biological variability of field-collected organisms.

In Pb exposed larvae an inhibition of Ca uptake at 24 hours (Figure 3-4b) coincided with lower concentrations of Ca at the same time point (Figure 3-11a). This could be because inhibition of Ca uptake is occurring before 24 hours (when uptake measurements are performed) and by the 24 hour mark has had a significant effect on Ca concentrations. A return to normal uptake levels at 60 and 72 hours (Figure 3-4b) results in a return of Ca concentrations to control levels for the remainder of the 96 hours from 72 hours onwards (Figure 3-11a). Perhaps the inhibition of Ca uptake seen at 84 and 96 hours (Figure 3-4b) would have resulted in lower Ca accumulation later in development beyond the last time point of the experiment (Figure 3-11a).

What is apparent from the Ca uptake flux experiments and the Ca concentration measurements in larvae over development is that larvae possess some capacity to recover from metal stress. This is because Ca uptake rates and Ca concentrations periodically return to normal at various time points over the metal exposure even though the larvae have evidently suffered toxic injury from metal stress. For example, by the end of 96 hours Pb exposed larvae had regained normal calcium levels (Figure 3-11a). This leads us to believe that toxic action of the metal might occur during the early hours of development, after which the larvae undergo some damage repair. However, this does not exclude the fact that irreversible damage to development might be occurring during early

key developmental stages, which might not be apparent through measuring Ca levels at 72 hours of development.

Pb and Zn accumulation over a range of exposure concentrations

The capacity of the larvae to regulate metal toxicity is also evident from our experiments performed in which larvae exposed to a range concentrations of Zn, which only started to accumulate significant amounts of the metal at 320 µg Zn/L (Figure 3-2c), before which metal accumulation was the same at all lower exposure concentrations. This was not true of Pb, which accumulated in the larvae at all concentrations in earlier research performed on *S. purupuratas* by this lab. Zn, as opposed to Pb is an essential metal and therefore the larvae might have some increased capacity to regulate its accumulation. Evidence of regulation of the uptake of essential ions is seen in *P. lividus* a similar species of sea urchin, where Zn displayed a similar threshold level in its accumulation (Radenac, 2001). DMT1 is a membrane protein responsible for the cellular uptake of essential divalent cations (Ballatori, 2002). In the case of Zn exposed larvae, expression of this protein may be down-regulated as a compensatory mechanism employed by the larvae to reduce the amount of metal that is taken up via this route. This downregulation although limiting Zn accumulation, may have negative implications on the uptake of other essential metals required by the urchin larvae.

Metal accumulation results have importance in the development of BLMs as there is a clear link between metal accumulation and metal toxicity (Meyer et al., 1999). The LA50 is defined as the amount of metal accumulation, which is associated with 50% mortality. While the dissolved metal EC50 varies with water chemistry, it always corresponds with the same metal accumulation (LA50) at the biotic ligand which is constant regardless of water chemistry (Meyer et al., 1999). Therefore the LA50 is a critical measure of toxicity.

Pb and Zn accumulation over development

In the current research one concentration of Zn (106 µg/L) was chosen for the exposure. This concentration was lower than the exposure concentrations at which metals start to accumulate in the larvae exposed to a range of Zn concentrations (Figure 3-2c). It is interesting to note that although Zn did not accumulate in the larvae by the end of 96 h, toxic effects of the metal were observed at this exposure concentration (106 µg/L) as is evident from the inhibition of Ca uptake (Figure 3-5b) and accumulation (Figure 3-10a).

Pb (54 µg/L) exposure resulted in significant accumulation of the metal at all time points over the first 96 h of development. Accumulation of the metal followed a step-wise pattern with no evidence of the larvae regulating its uptake (Figure 3-12b).

ATPase activity

Transport of metals across plasma membranes of cells can occur through ATP-dependent processes, either through transfer by proteins intrinsic to the cell membrane with specificity for metal ions, or through receptor mediated-endocytosis. Once the metal is inside the cell it binds to ligands, and this binding maintains a concentration gradient down which metals can continue to travel into the cell and cause toxic effects, such as inactivation of enzymes (Viarango et al., 1996). Inhibition of ATPase enzymes has been associated with metals binding with the sulfhydral groups of these enzymes, which alters the transport of ions into cells (Pivovarova and Laerspetz, 1996). Inactivation of these enzymes has also been attributed to damage caused by reactive oxygen species (Stark, 2005).

Much of the toxicity caused by metals may be due to oxidative stress from free radical production catalyzed by metals. Reactive oxygen species compromise the integrity of the biological membranes either by interacting with cellular machinery or by oxidizing macromolecules of the cell membrane, causing a variety of functional alterations (Stark, 2005). Lipid peroxidation resulting in loss of function of membrane enzymes, is one such example of damage caused by reactive oxygen species. Reactive oxygen species alter the fatty acid profile of the cell membrane, thus in turn disrupting modulation of membrane-bound ATPases whose activity depends on membrane phospholipids (Reddy and Philip, 1992)

Mg^{2+}, Ca^{2+} and Na^{+}/K^{+} ATPases function to control membrane permeability by transporting ions across the cell membrane, which in turn modulate cell volume and osmotic pressure (Reddy and Philip, 1992). Alteration of ATPase enzyme activity is a sensitive measure of general cell health and therefore an effective biomarker of stress from toxicants (Sancho et al., 2003).

Our current research suggests inhibition of Ca^{2+} ATPase activity as a possible mechanism of toxicity of chronic Zn exposure. Ca^{2+} ATPase activity was significantly lower than controls at 24, 36 and 48 h over 72 h of development (Figure 3-6b). Pb exposure larvae however showed an upregulation of Ca^{2+} ATPase at 48 h and 60 h (Figure 3-6a) . This could have contributed to the return to normal of Ca uptake rates at 60 and 72 h (Figure 3.4a) and normal Ca levels at 48 h and after 72 h (Figure 3-11a).

Conclusion

Our research suggests that the toxicity of Pb and Zn stems primarily from a disruption of Ca homeostasis during early development of embryos and larvae. However, the acute metal challenge experiments demonstrated that this was not by a direct competition of Pb or Zn for the Ca uptake mechanism. Interestingly, the larvae display some capacity for recovery from toxicant stress as is evident from the return to normalcy of Ca uptake rates, Ca accumulation and Ca ATPase activity periodically over development. Although there is some damage repair evident, other toxic effects may still be apparent from measuring biological endpoints not analyzed in the present study. Additionally, compensatory mechanisms employed to combat metal toxicity, such us the

downregulation of DMT1 expression mentioned earlier, may also have effects on the health of urchins later in their lives.

Toxic effects of metal exposure seen at earlier time points during development were sometimes not apparent at 72 h of development, the endpoint of standard toxicity tests used routinely in environmental monitoring. We propose measuring endpoints of toxicity periodically over early development as a more effective way of studying the toxic stress of contaminants.

Figure 3-1 Embryo survival over a range of salinities. Values are means ± SEM (N = 5).

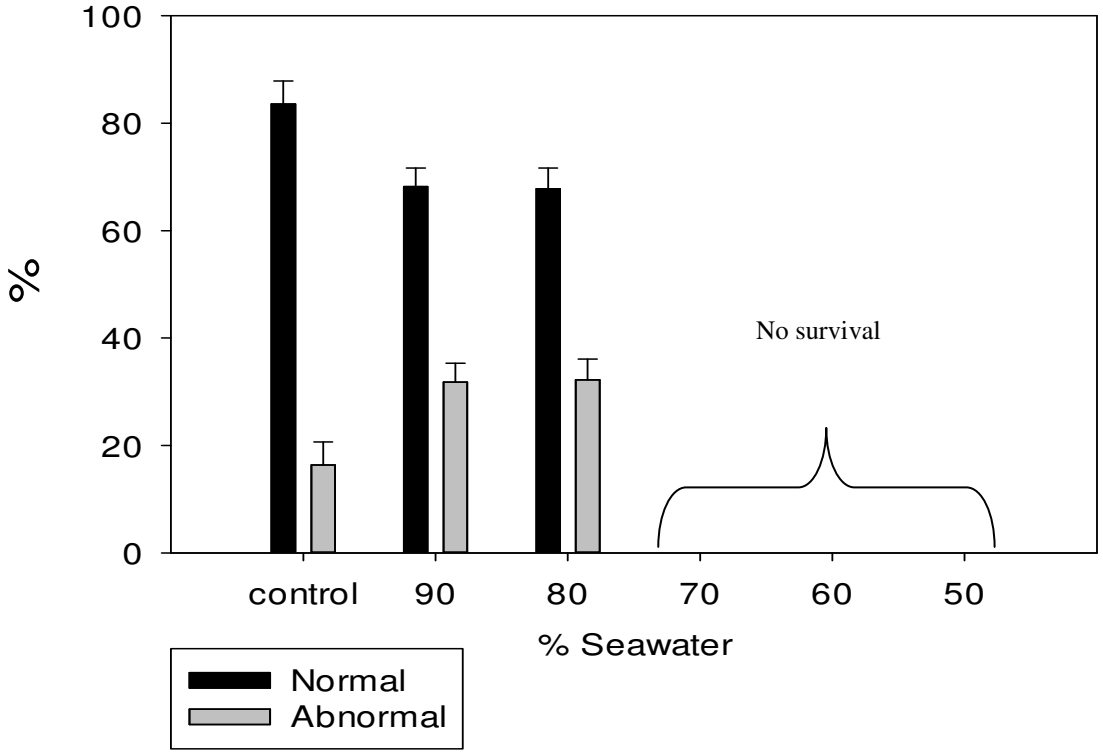
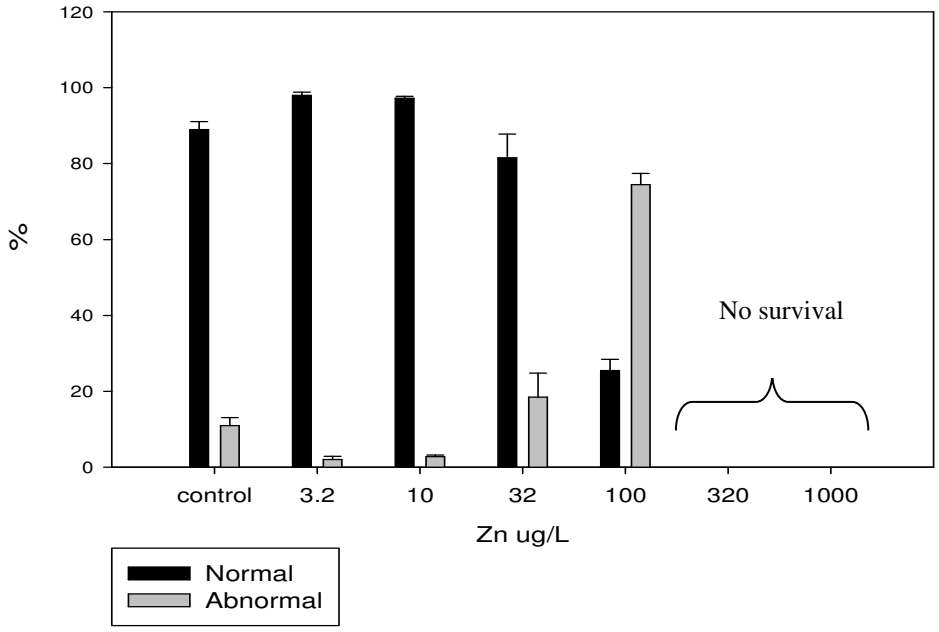
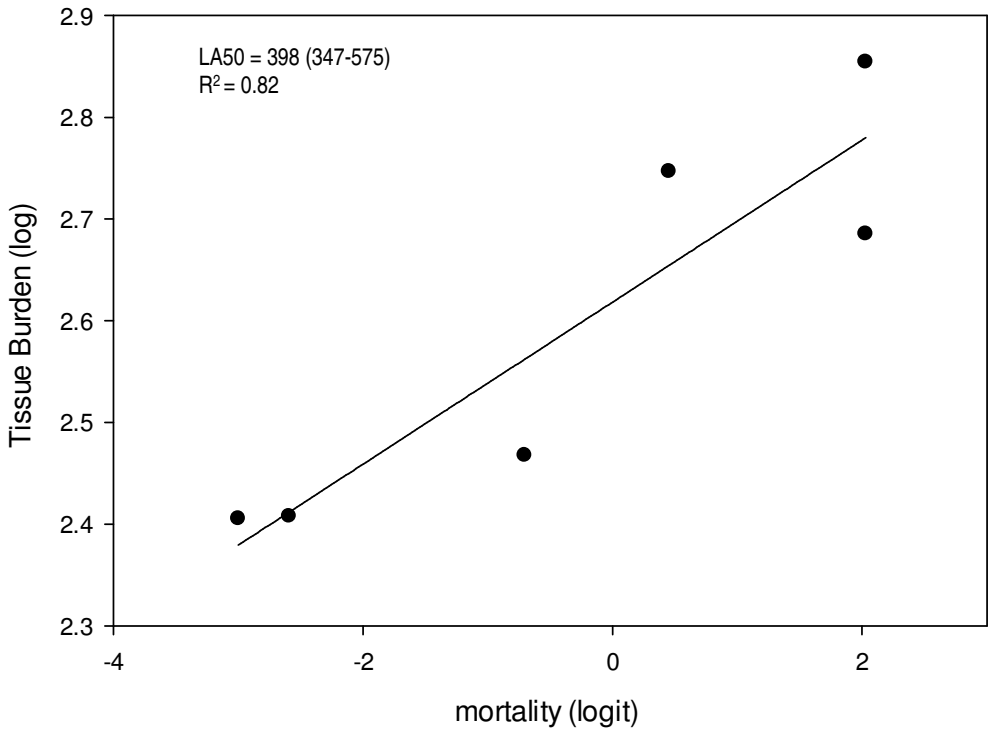

100
80
60
40
20
0
%
No survival
control
90
80
70
60
50
% Seawater
Normal
Abnormal

Figure 3-2 a) Percentage normal versus abnormal embryos over a range of Zn concentrations **b)** Relationship between whole body Zn burden and mortality **c)** Whole body metal accumulation at 72 hours of development in larvae exposed to a range of concentrations of Zn. Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc Values are means ± SEM (N = 5).

a


120
100
80
60
40
20
0
%
No survival
control
3.2
10
32
100
320
1000
Zn ug/L
Normal
Abnormal

b


LA50 = 398 (347-575)
R² = 0.82
Tissue Burden (log)
mortality (logit)
2.9
2.8
2.7
2.6
2.5
2.4
2.3
-4
-2
0
2

c

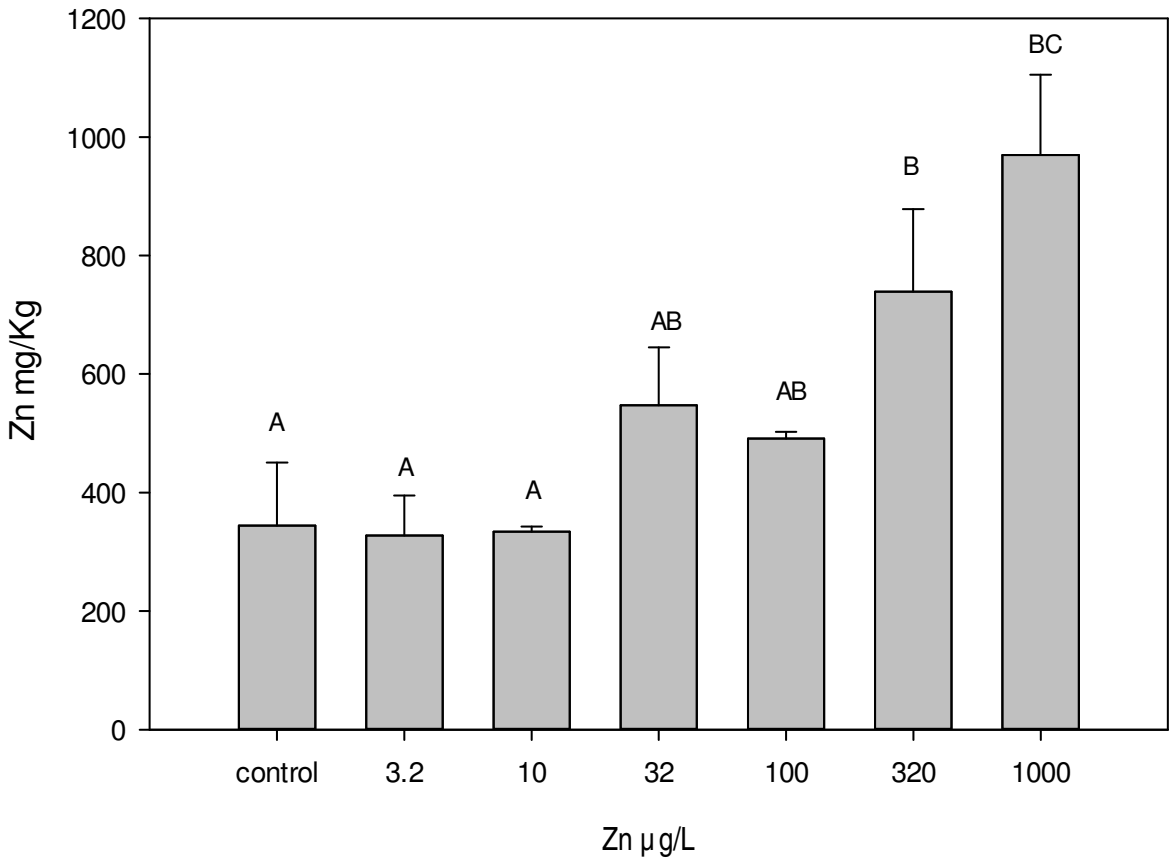
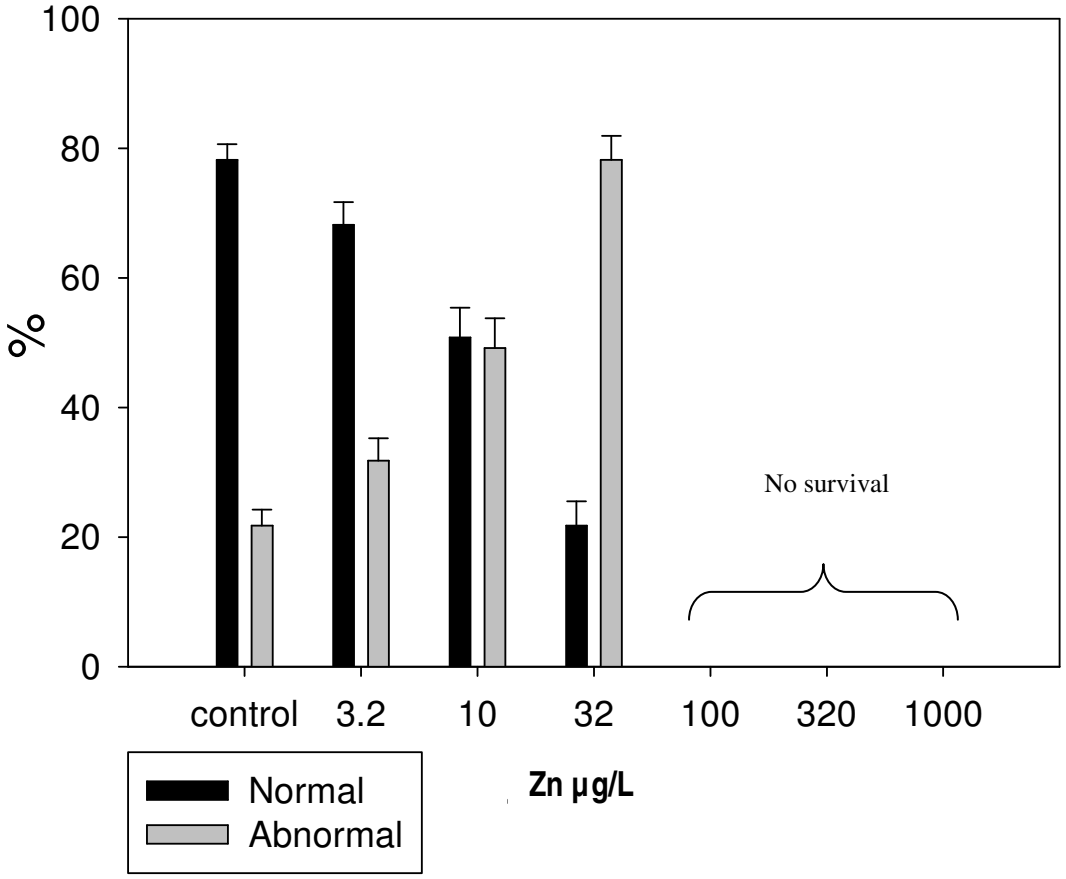

1200
1000
800
600
400
200
0
Zn mg/Kg
A
A
A
AB
AB
B
BC
control
3.2
10
32
100
320
1000
Zn µg/L

Figure 3-3. Percentage normal versus abnormal embryos over a range of Zn concentrations with **a)** 3mg/L Bamfield inshore DOC and **b)** 12 mg/L Bamfield inshore DOC. Values are means ± SEM (N = 5).
NB. Data for Nordic reservoir DOC not shown as there was no survival in any treatment.

a


100
80
60
40
20
0
%
No survival
control
3.2
10
32
100
320
1000
Zn µg/L
Normal
Abnormal

b

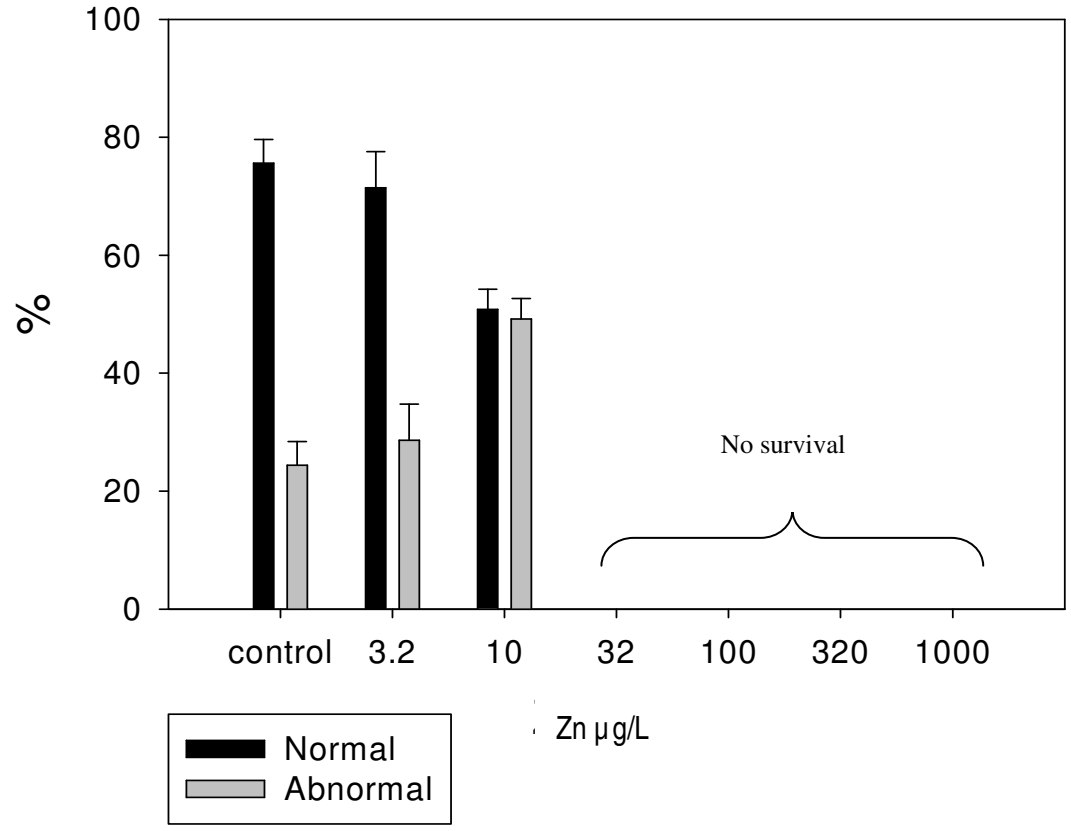
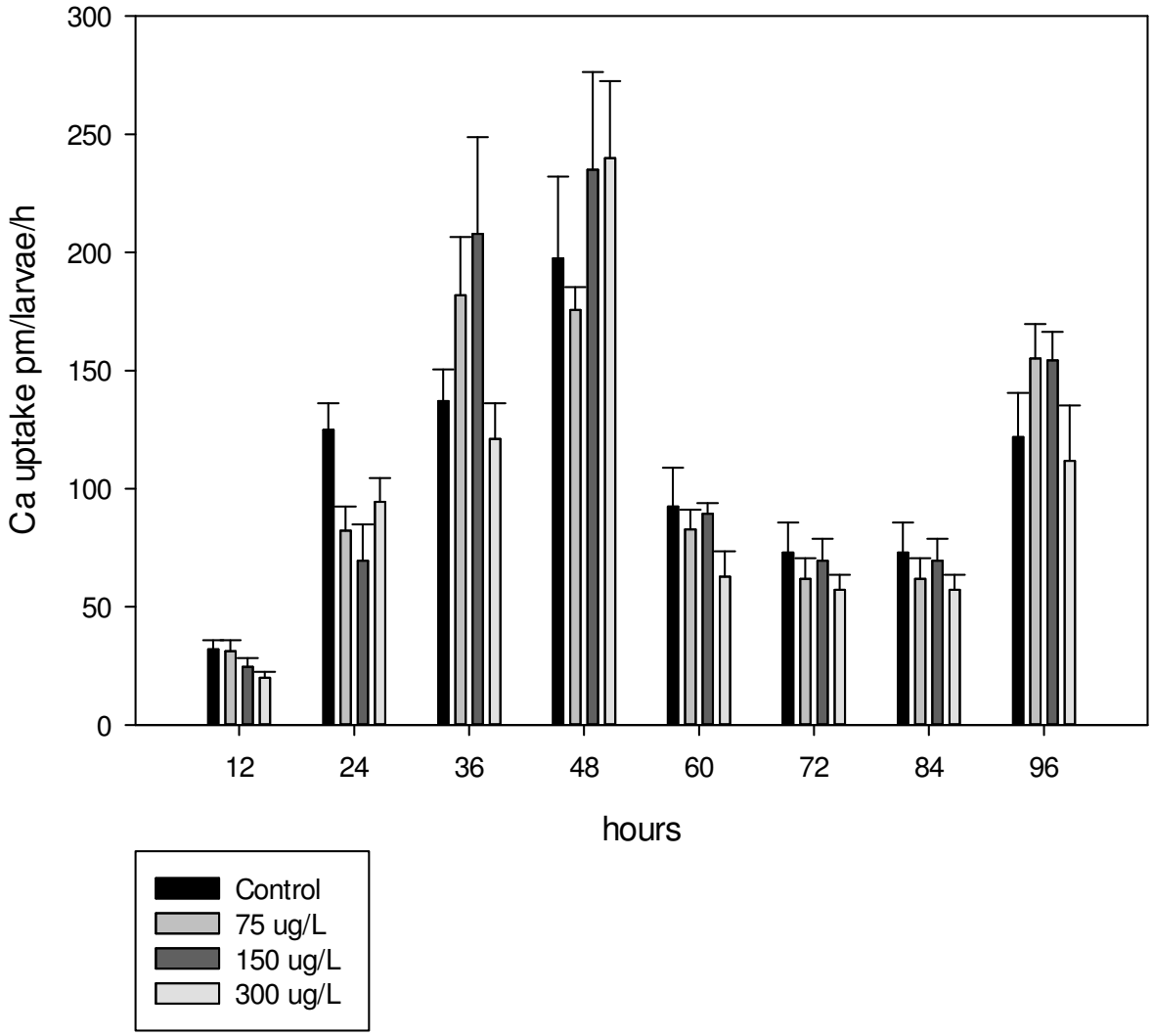

100
80
60
40
20
0
%
No survival
control
3.2
10
32
100
320
1000
Zn µg/L
Normal
Abnormal

Figure 3-4. Ca uptake rates every 12 hrs over the first 96 hours larval development in **a)** acutely Pb exposed larvae and **b)** chronically Pb (60 µg/L) exposed larvae. An asterisk (*) indicates a significant difference from control levels as determined with a Student's t-test ($P<0.05$). Values are means ± SEM ($N = 6$).

a


300
250
200
150
100
50
0
Ca uptake pm/larvae/h
12
24
36
48
60
72
84
96
hours
Control
75 ug/L
150 ug/L
300 ug/L

b

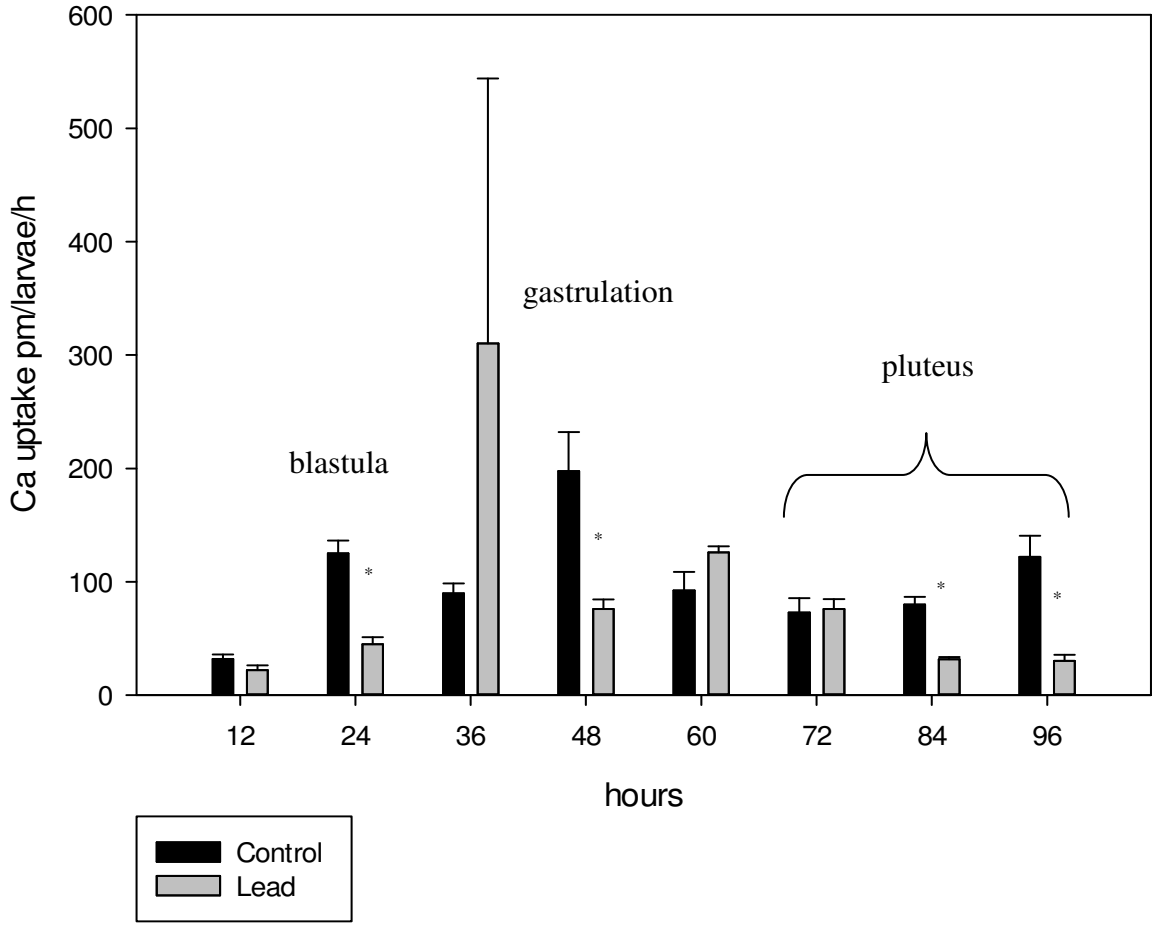
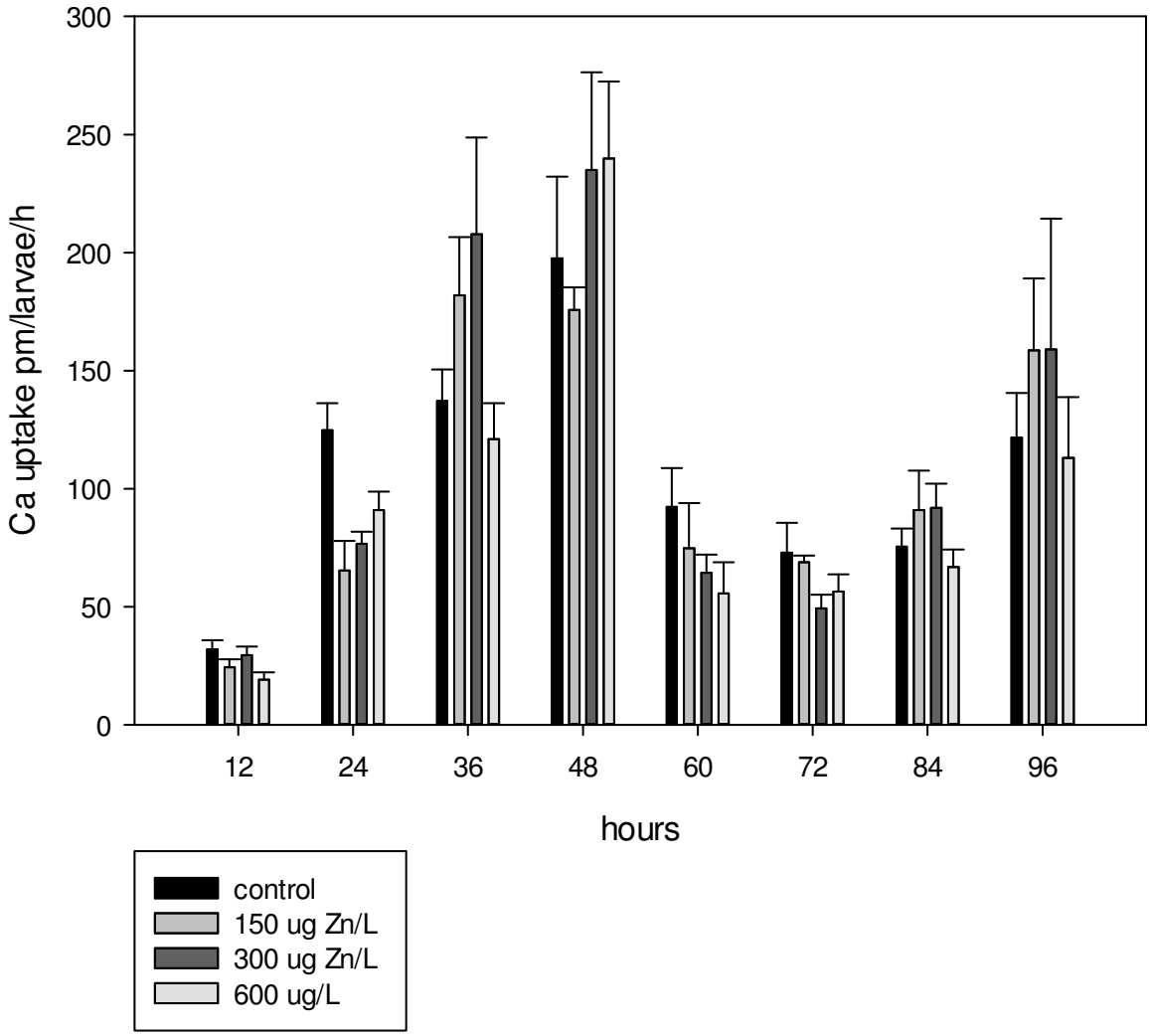

600
500
400
300
200
100
0
Ca uptake pm/larvae/h
blastula
gastrulation
pluteus
*
*
*
*
12
24
36
48
60
72
84
96
hours
Control
Lead

Figure 3-5 Ca uptake rates every 12 hrs over the first 96 hours larval development in **a)** acutely Zn exposed larvae and **b)** chronically Zn (139 µg/L) exposed larvae. An asterisk (*) indicates a significant difference from control levels as determined with a Student's t-test ($P<0.05$). Values are means ± SEM ($N = 5$).

a


300
250
200
150
100
50
0
Ca uptake pm/larvae/h
12
24
36
48
60
72
84
96
hours
control
150 ug Zn/L
300 ug Zn/L
600 ug/L

b

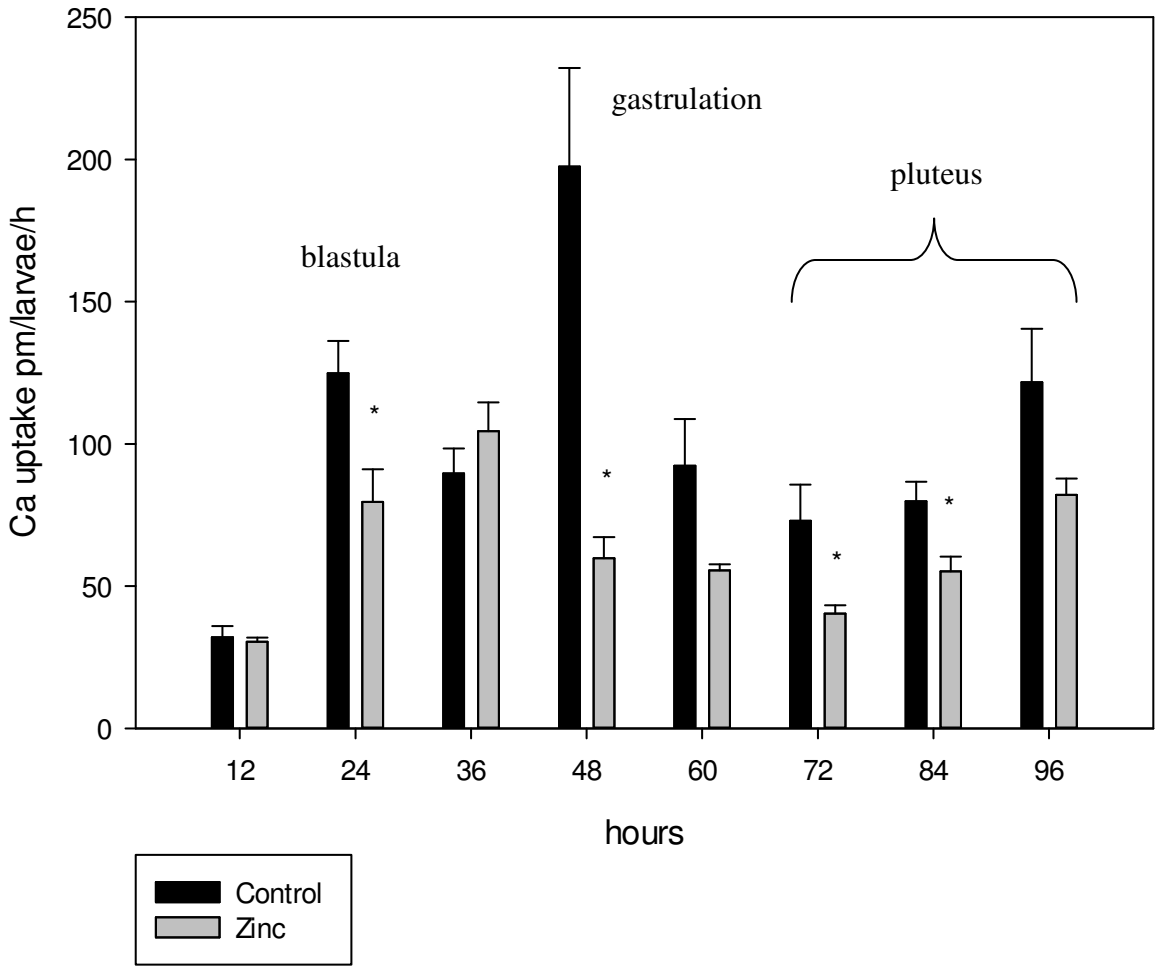
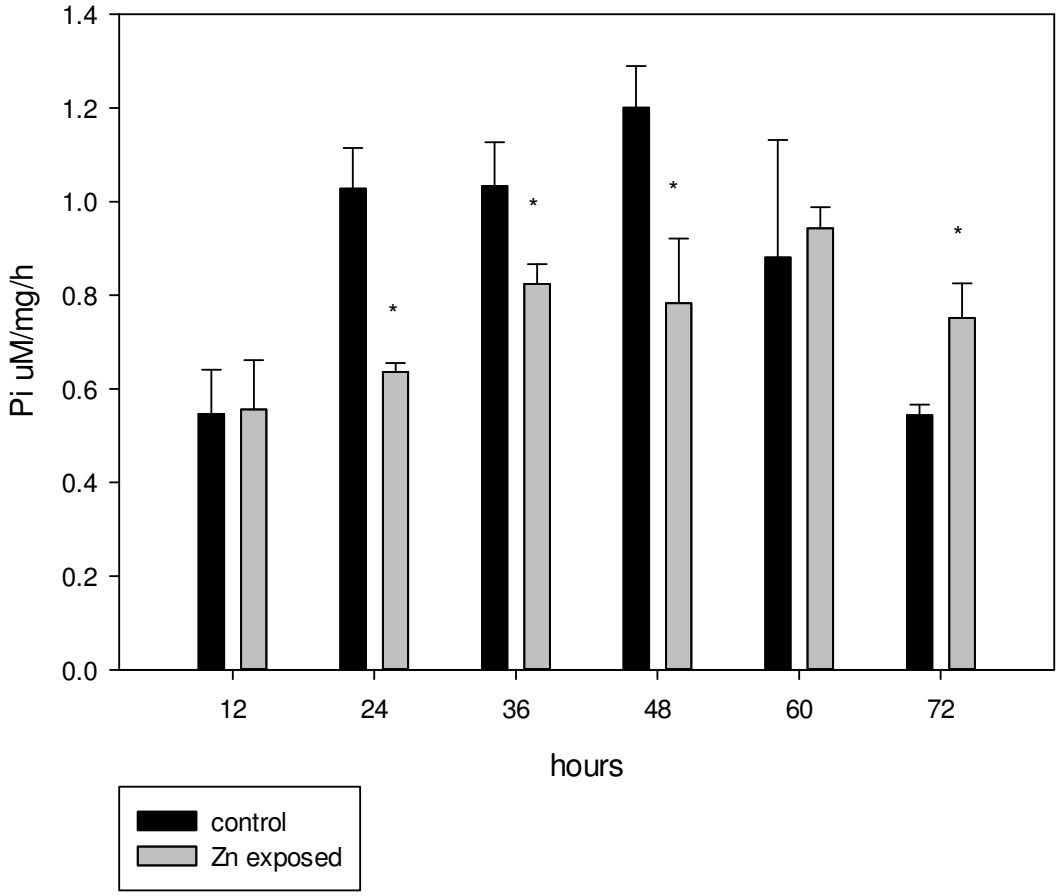

250
200
150
100
50
0
Ca uptake pm/larvae/h
blastula
gastrulation
pluteus
*
*
*
*
12
24
36
48
60
72
84
96
hours
Control
Zinc

Figure 3-6 Ca ATPase activity over 72 h of development in **a)** Pb exposed (60 µg/L) and **b)** Zn exposed (139 µg/L). An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's t-test ($P<0.05$). Values are means ± SEM ($N = 5$).

a


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi uM/mg/h
12
24
36
48
60
72
hours
control
Zn exposed
*
*
*
*

b

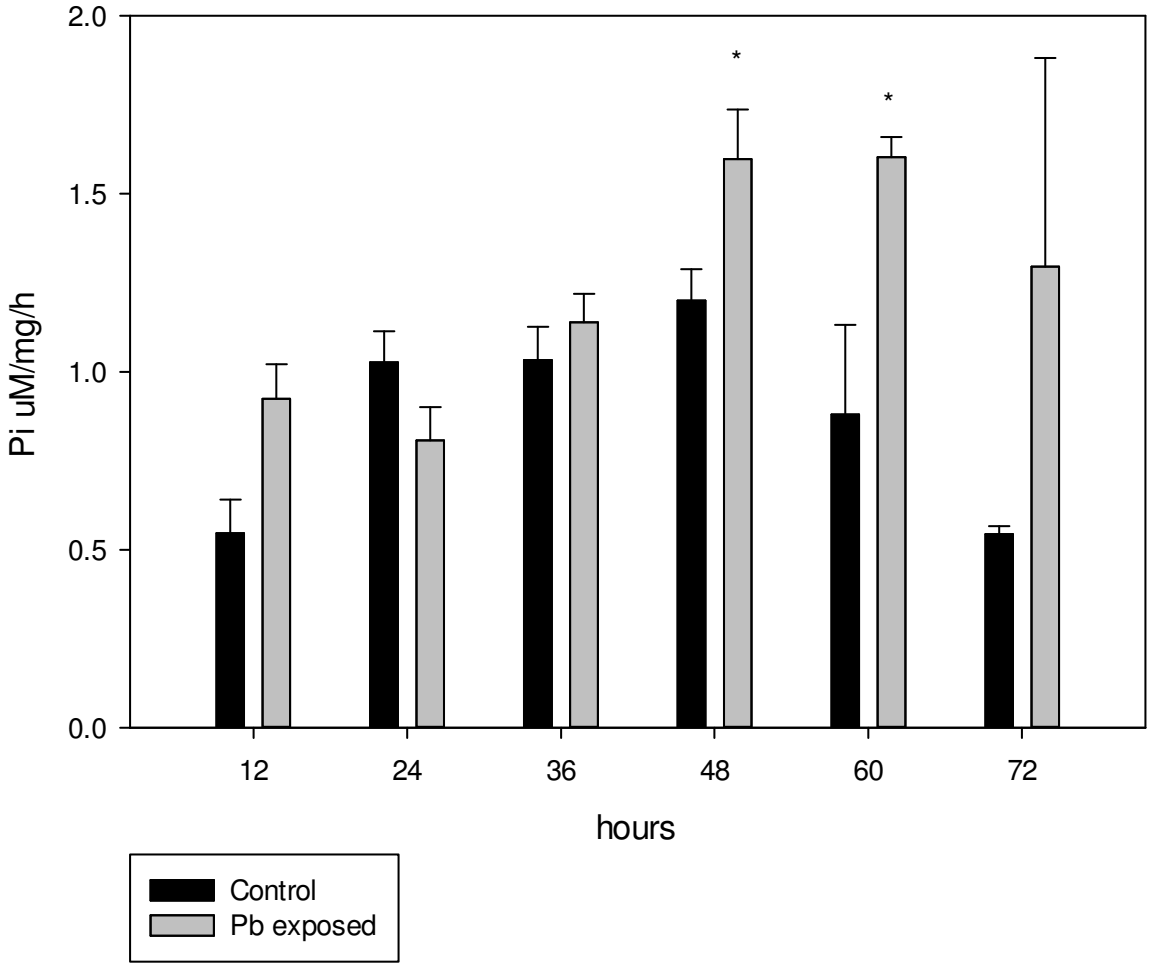
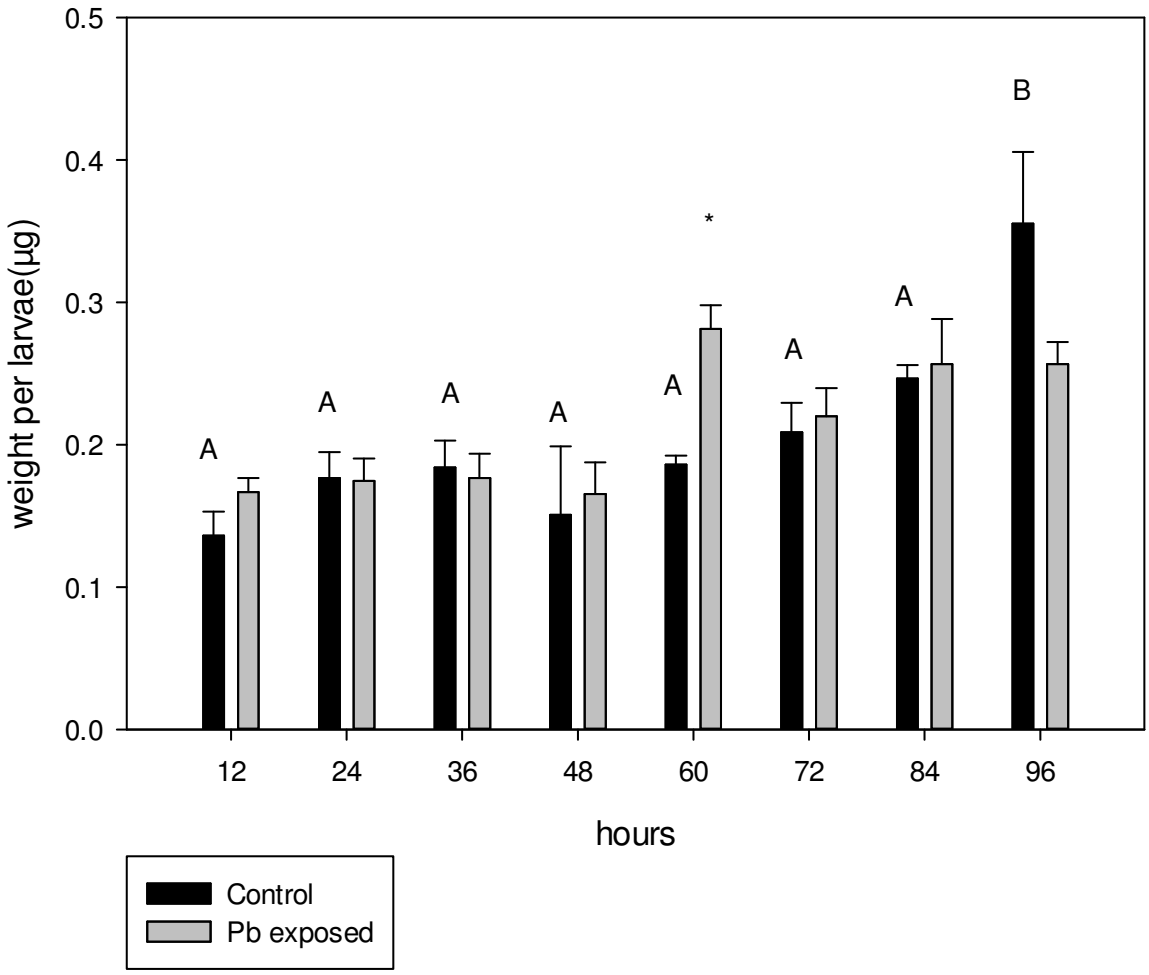

2.0
1.5
1.0
0.5
0.0
Pi uM/mg/h
*
*
12
24
36
48
60
72
hours
Control
Pb exposed

Figure 3-7 Larval weights every 12 hrs over the first 96 hours larval development in **a)** chronically Pb (60 µg/L) exposed larvae and **b)** chronically Zn (139 µg/L). An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's t-test ($P<0.05$). Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Letters of different cases indicate comparisons within treatments; upper case letters represent comparison between controls and lower case letters represent comparison between treatments. Values are means ± SEM ($N = 5$).

a


0.5
0.4
0.3
0.2
0.1
0.0
weight per larvae(µg)
A
A
A
A
A
*
A
A
B
12
24
36
48
60
72
84
96
hours
Control
Pb exposed

b

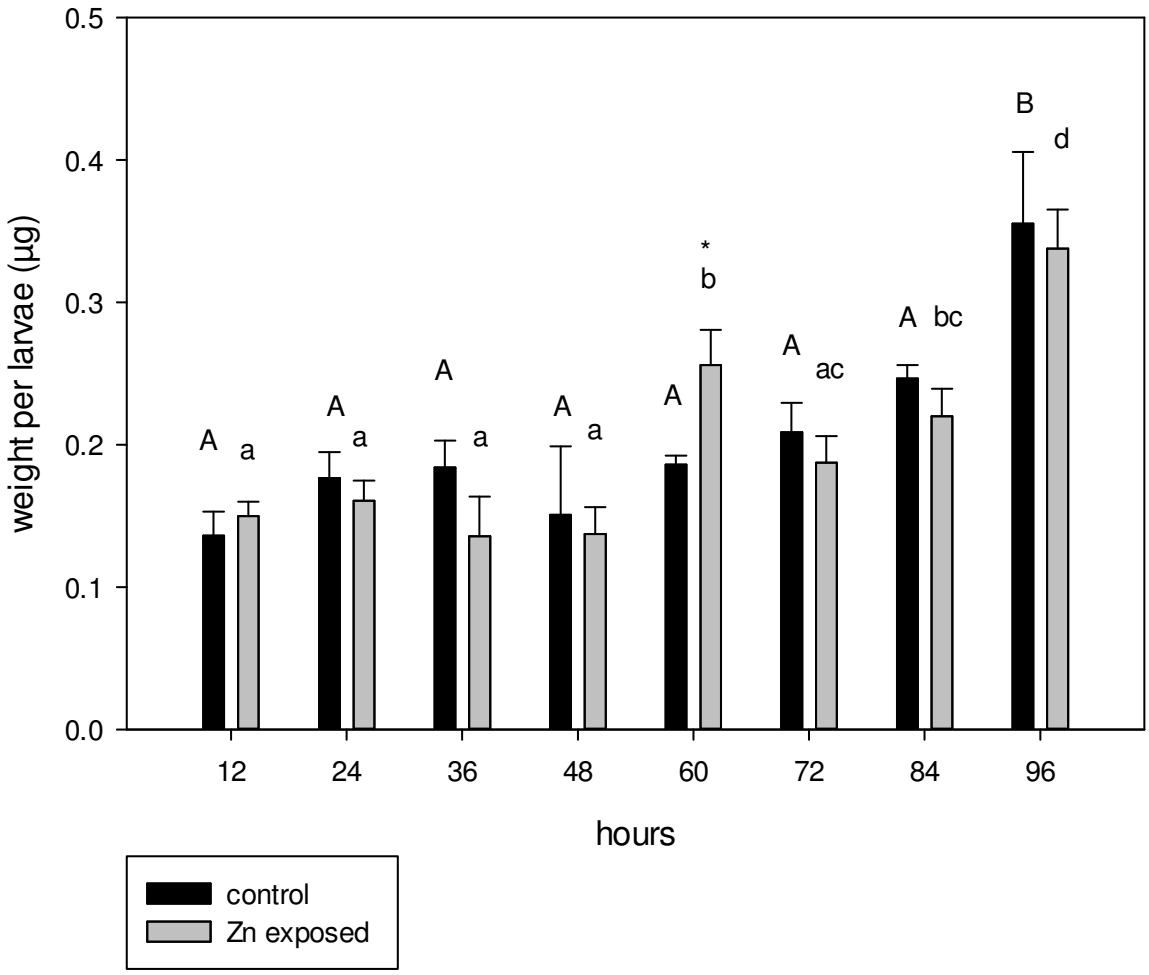
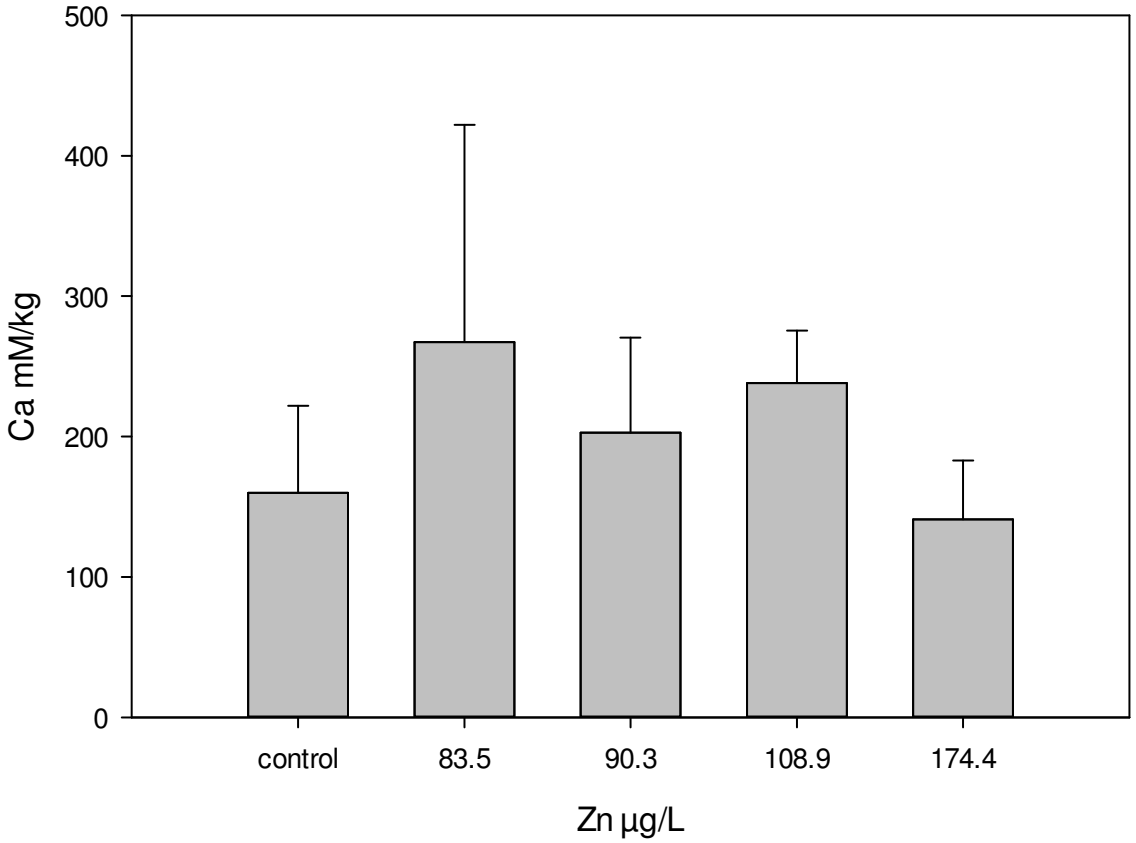
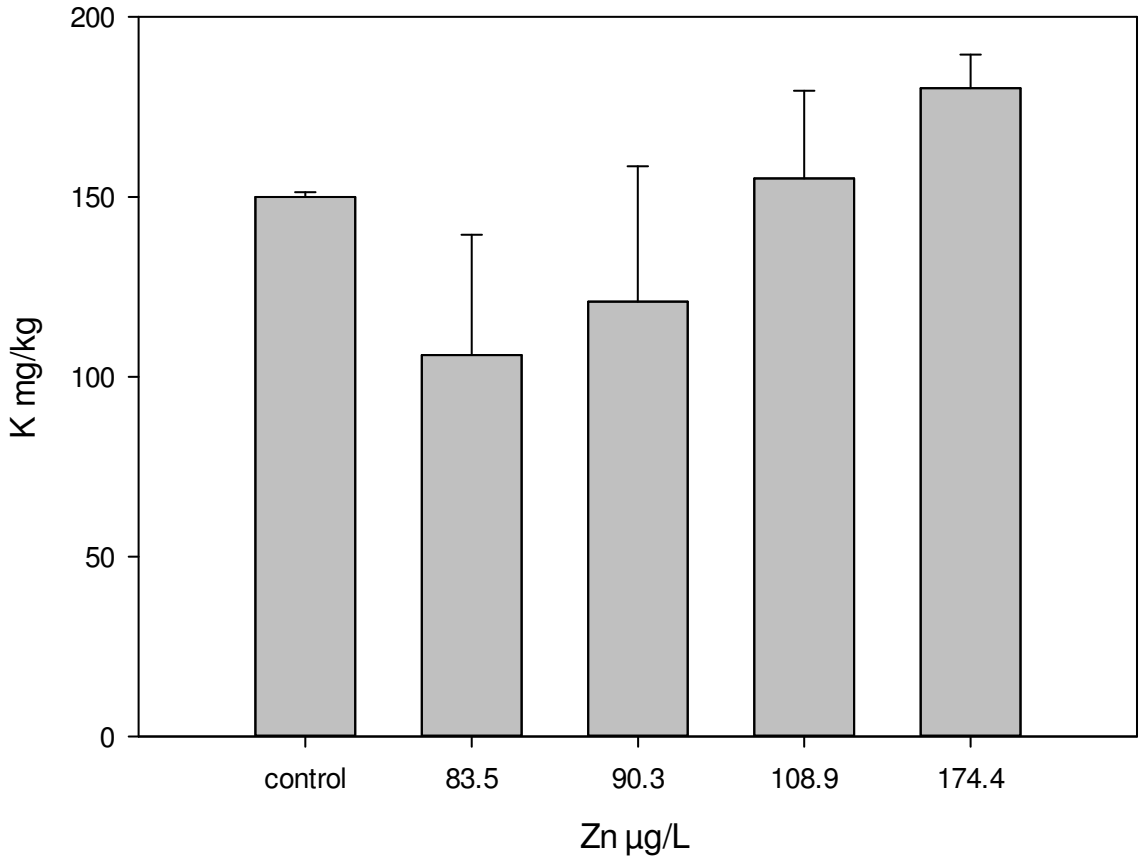

0.5
0.4
0.3
0.2
0.1
0.0
weight per larvae (µg)
A a
A a
A a
A a
A *b
A ac
A bc
B d
12
24
36
48
60
72
84
96
hours
control
Zn exposed

Figure 3-8 Whole body ion levels in larvae exposed to a Zn series **a)** Potassium **b)** Magnesium **c)** Sodium **d)** Calcium. Values are means ± SEM (N = 5).

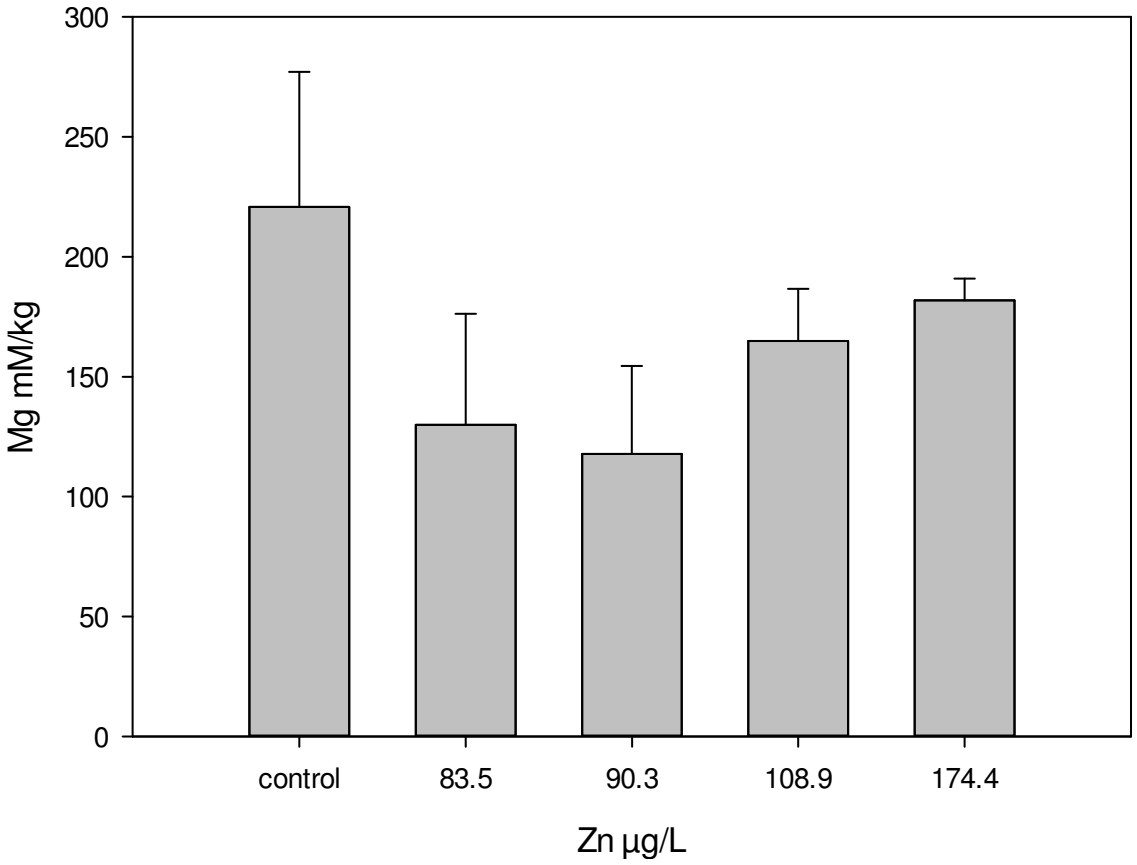
a



b



c


300
250
200
150
100
50
0
Mg mM/kg
control
83.5
90.3
108.9
174.4
Zn µg/L

d

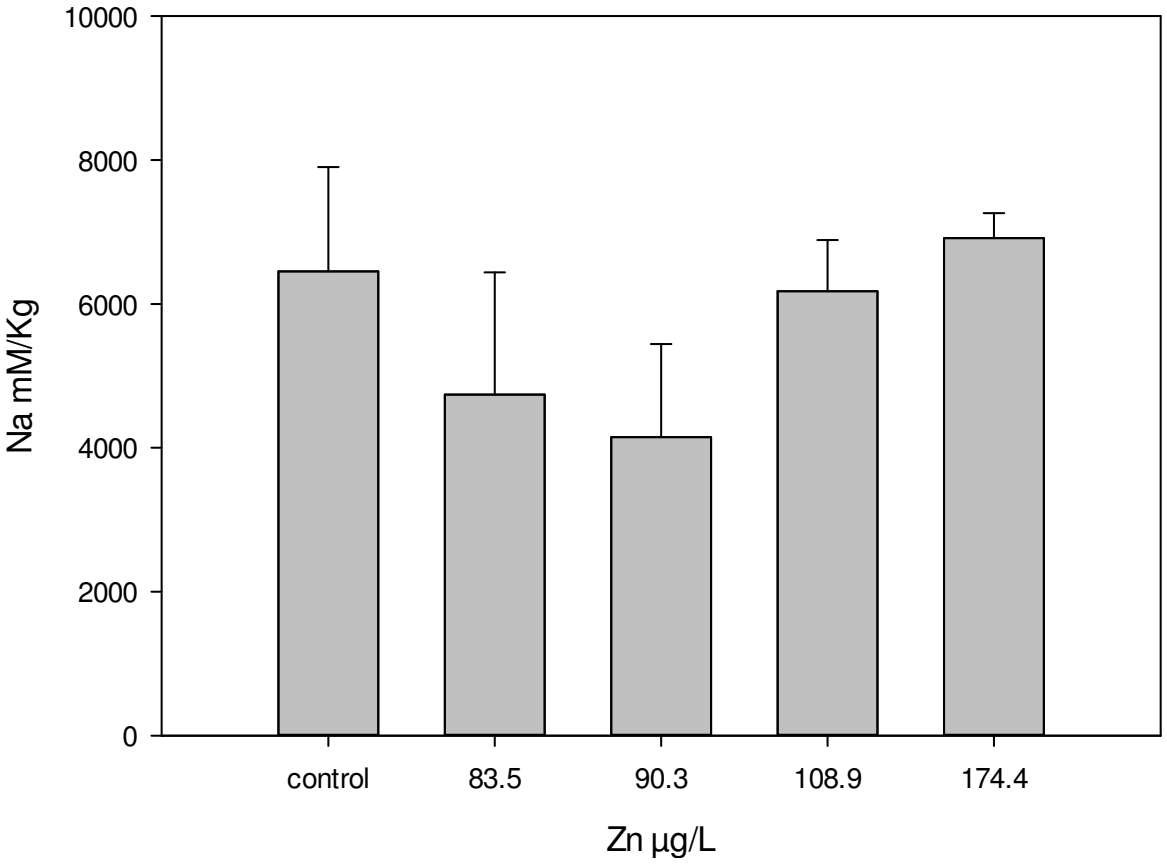
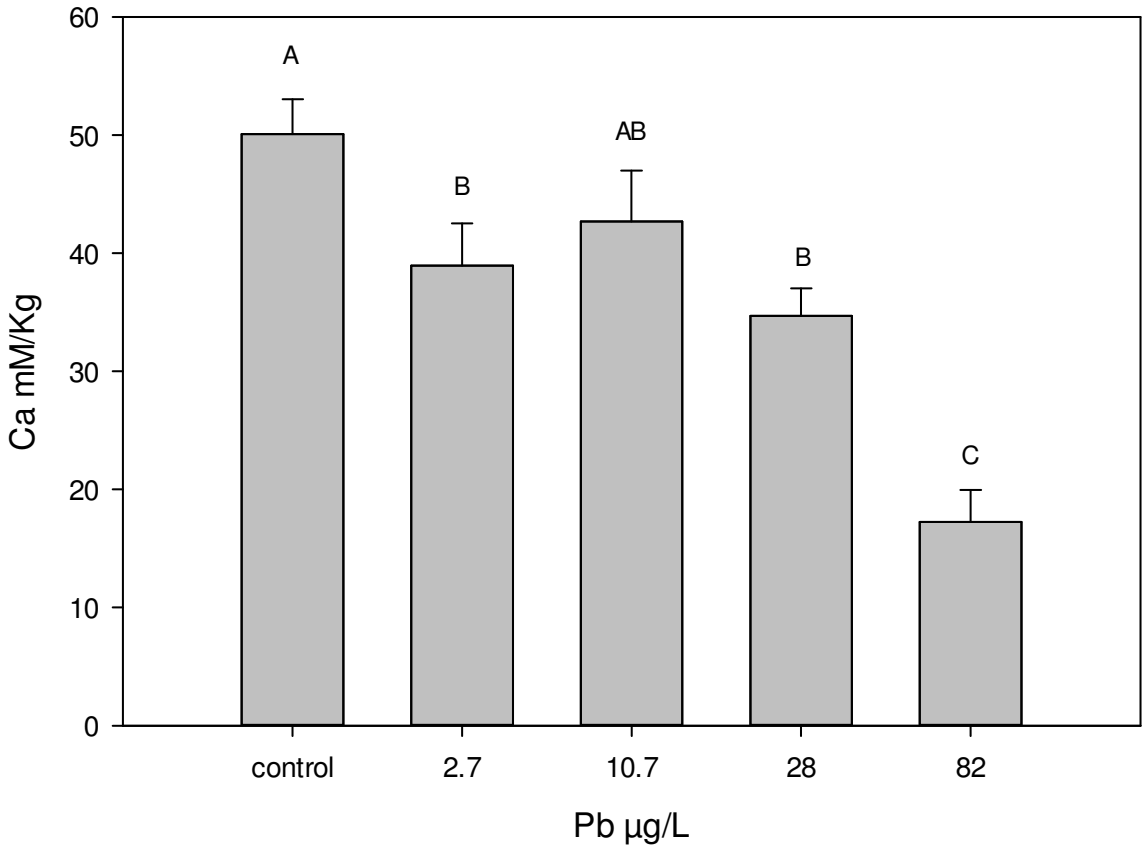
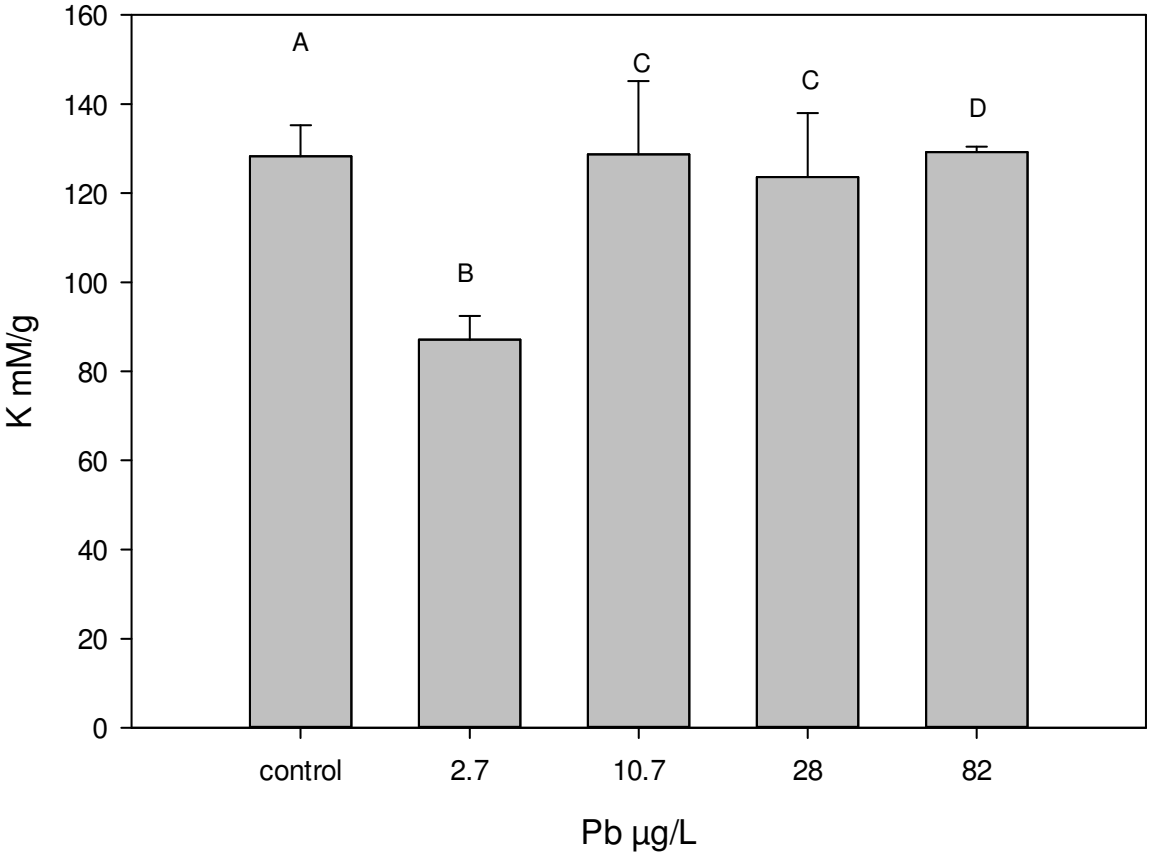

10000
8000
6000
4000
2000
0
Na mM/Kg
control
83.5
90.3
108.9
174.4
Zn µg/L

Figure 3-9 Whole body ion levels in larvae exposed to a Pb series **a)** Calcium **b)** Potassium **c)** Magnesium **d)** Sodium. Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Letters of different cases indicate comparisons within treatments. Values are means ± SEM (N = 5).

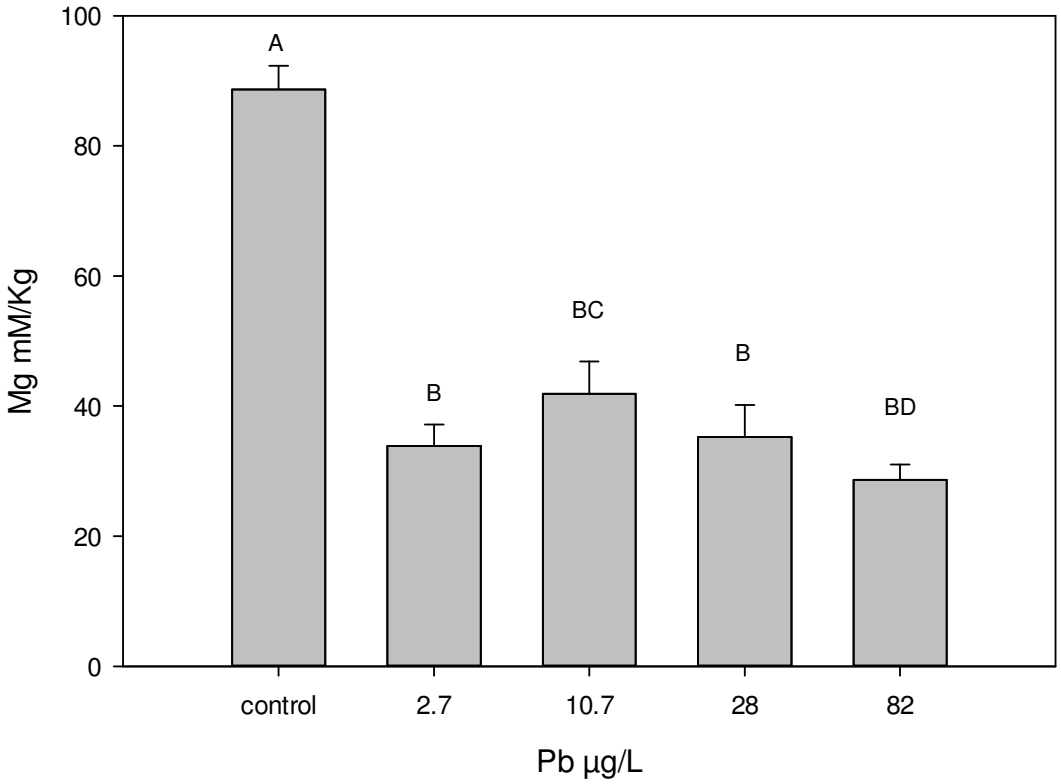
a



b


160
140
120
100
80
60
40
20
0
K mM/g
A
B
C
C
D
control
2.7
10.7
28
82
Pb µg/L

c



d

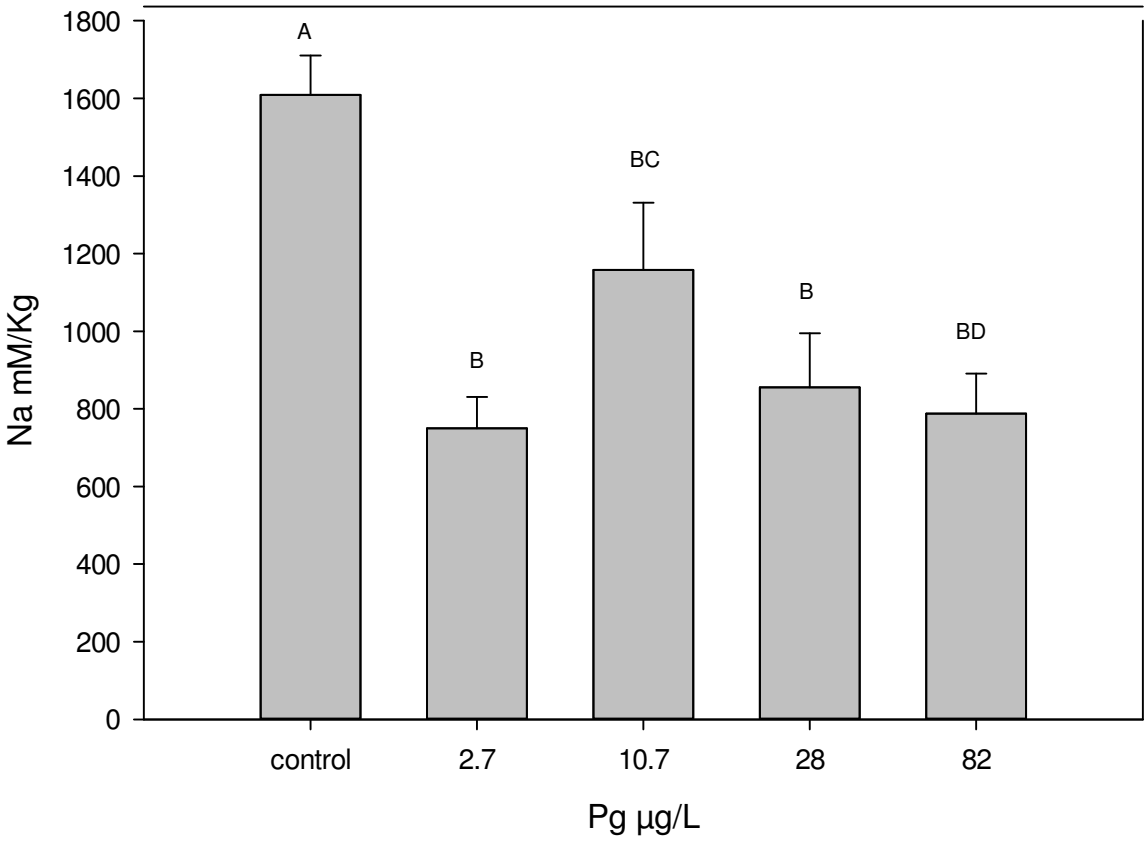
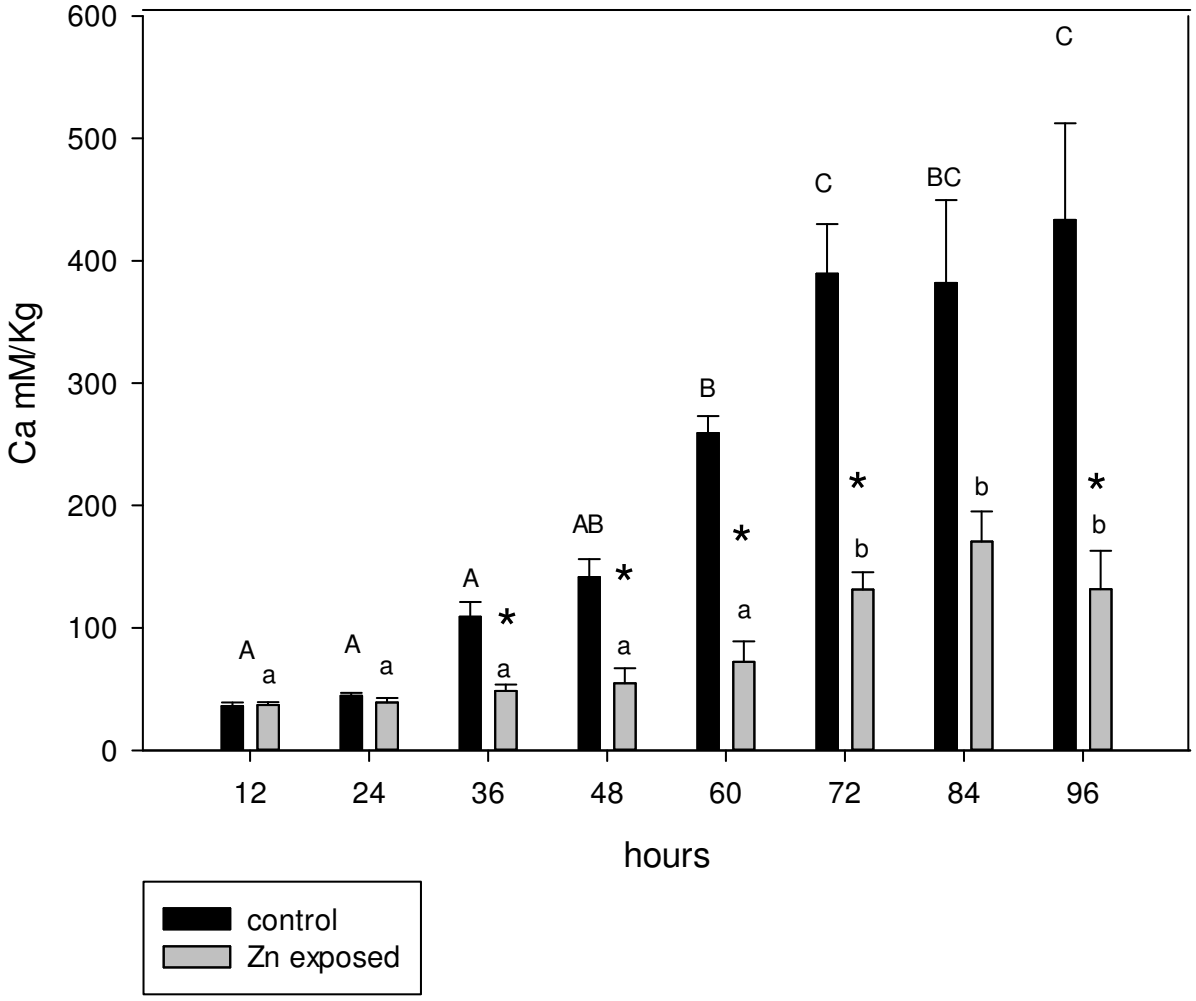
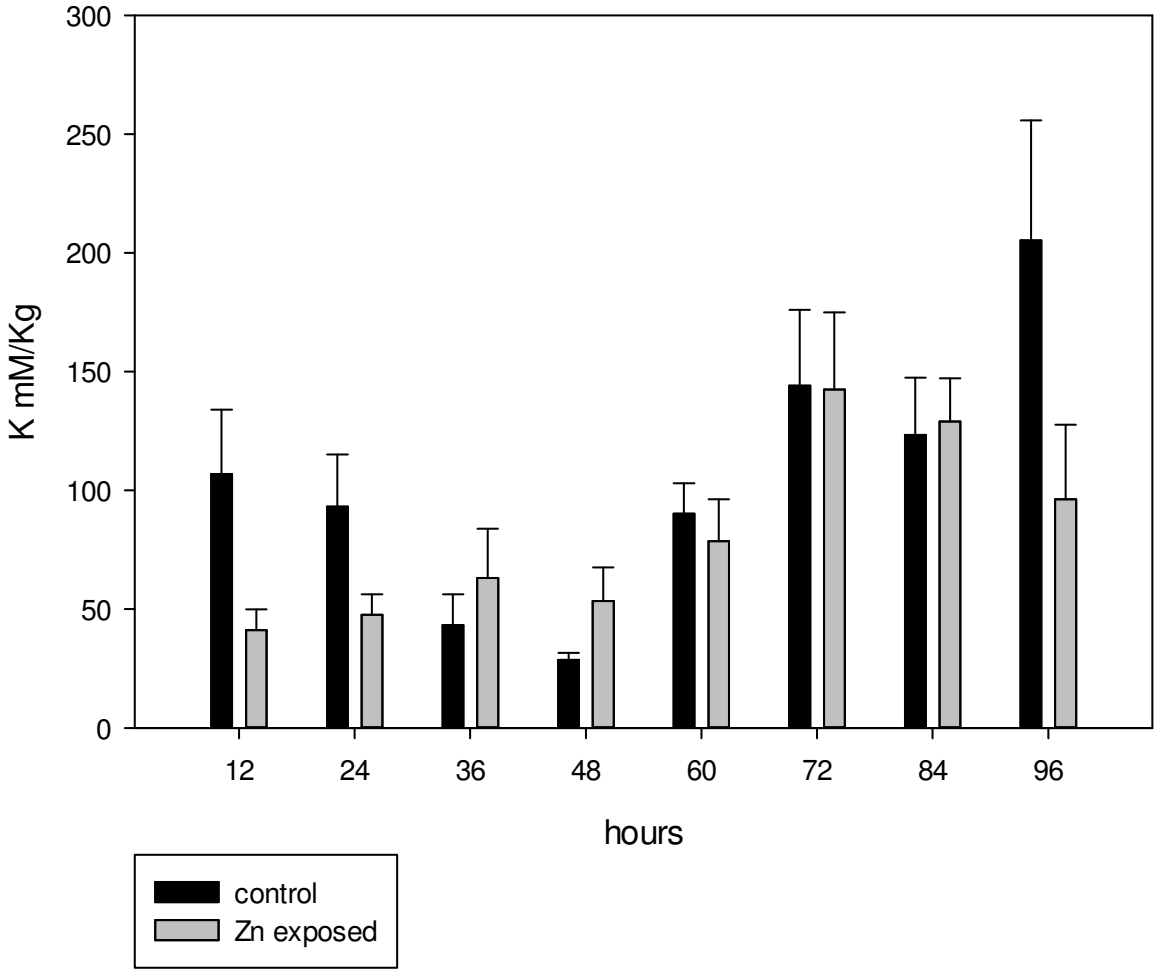

1800
1600
1400
1200
1000
800
600
400
200
0
Na mM/Kg
A
B
BC
B
BD
control
2.7
10.7
28
82
Pg µg/L

Figure 3-10 Whole body ion levels in larvae exposed to Zn (139 µg/L) over 96 hours of development **a)** Calcium **b)** Potassium **c)** Sodium **d)** Magnesium**.** An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's t-test ($P<0.05$). Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Letters of different cases indicate comparisons within treatments; upper case letters represent comparison between controls and lower case letters represent comparison between treatments. Values are means ± SEM ($N = 5$).

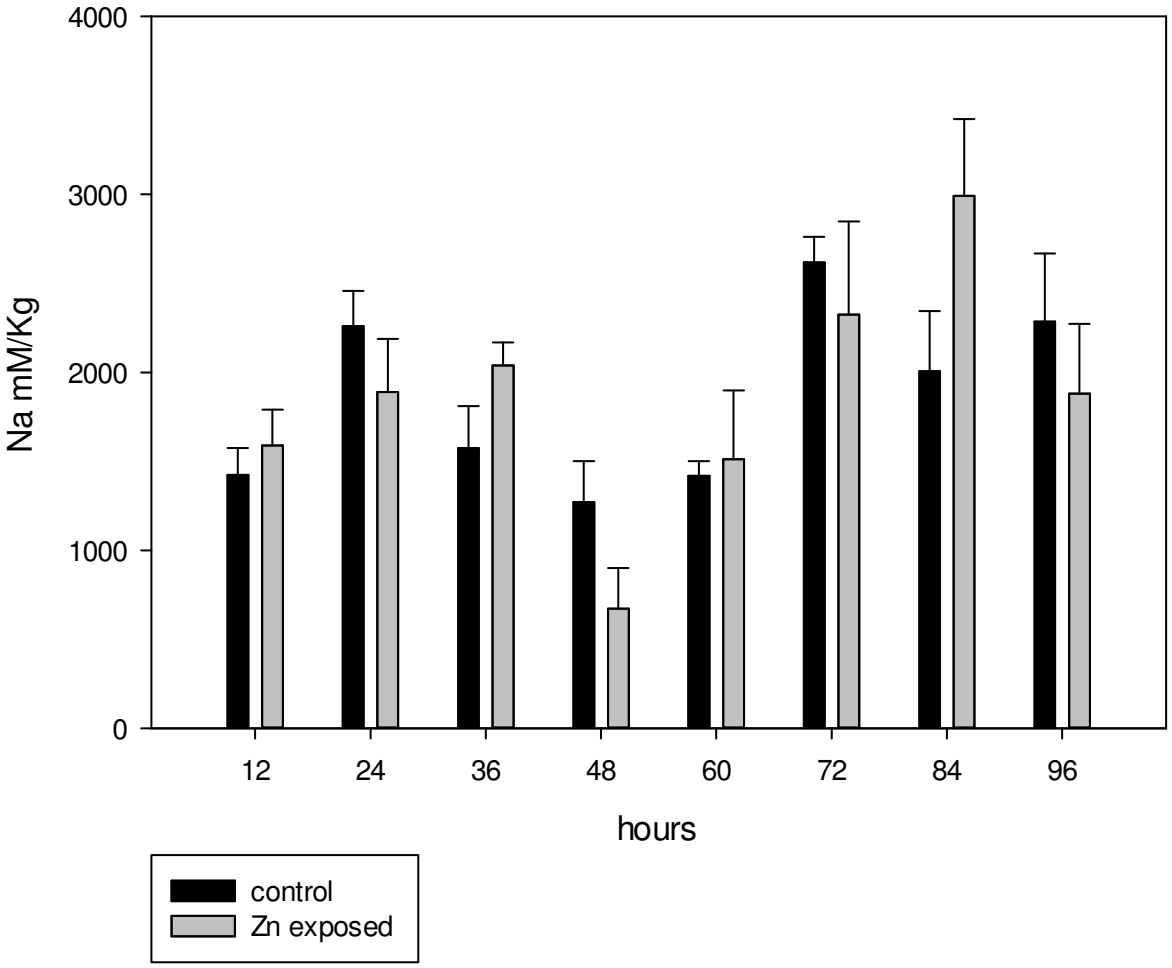
a


600
500
400
300
200
100
0
Ca mM/Kg
A
a
A
a
A
*
a
AB
*
a
B
*
a
C
*
b
BC
b
C
*
b
12
24
36
48
60
72
84
96
hours
control
Zn exposed

b


300
250
200
150
100
50
0
K mM/Kg
12
24
36
48
60
72
84
96
hours
control
Zn exposed

c


4000
3000
2000
1000
0
Na mM/Kg
12
24
36
48
60
72
84
96
hours
control
Zn exposed

d

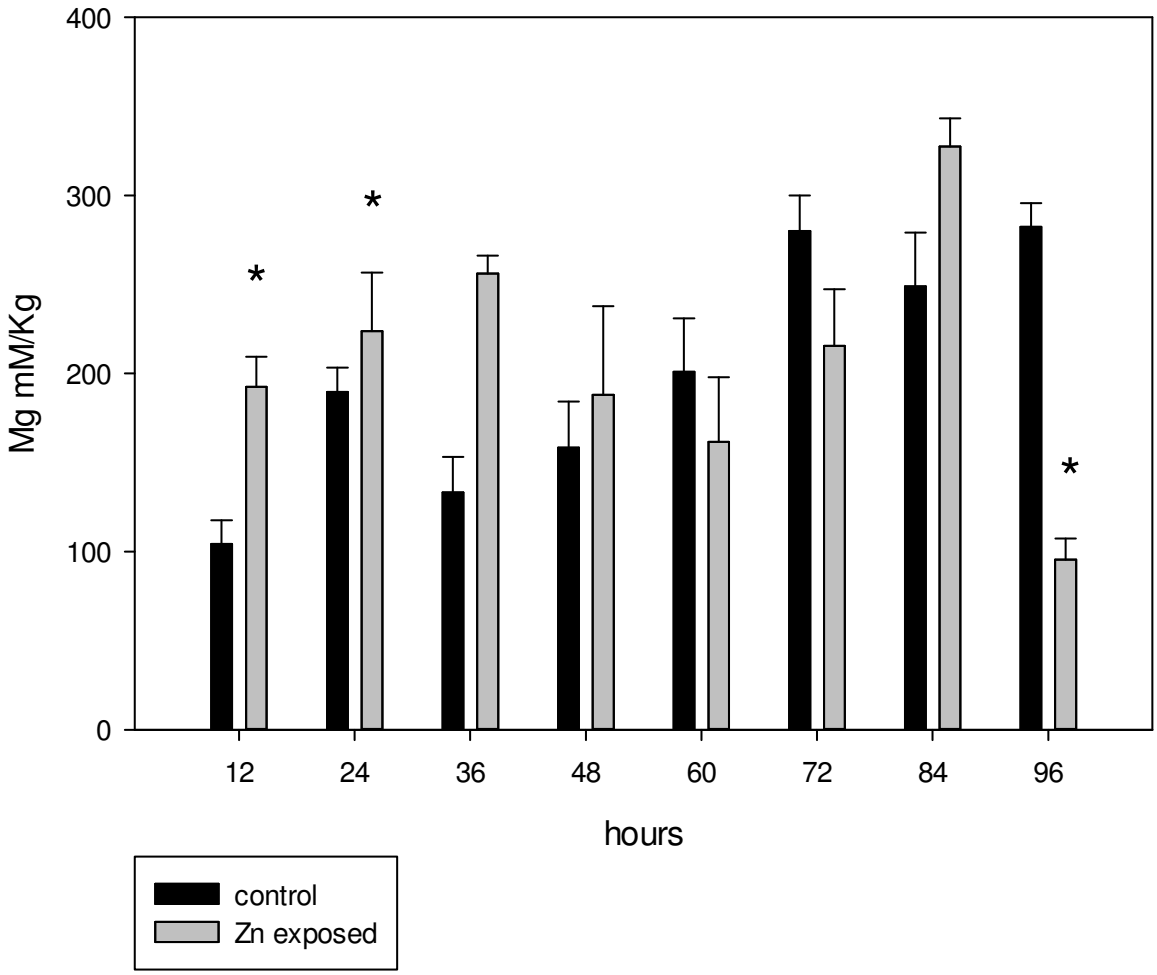
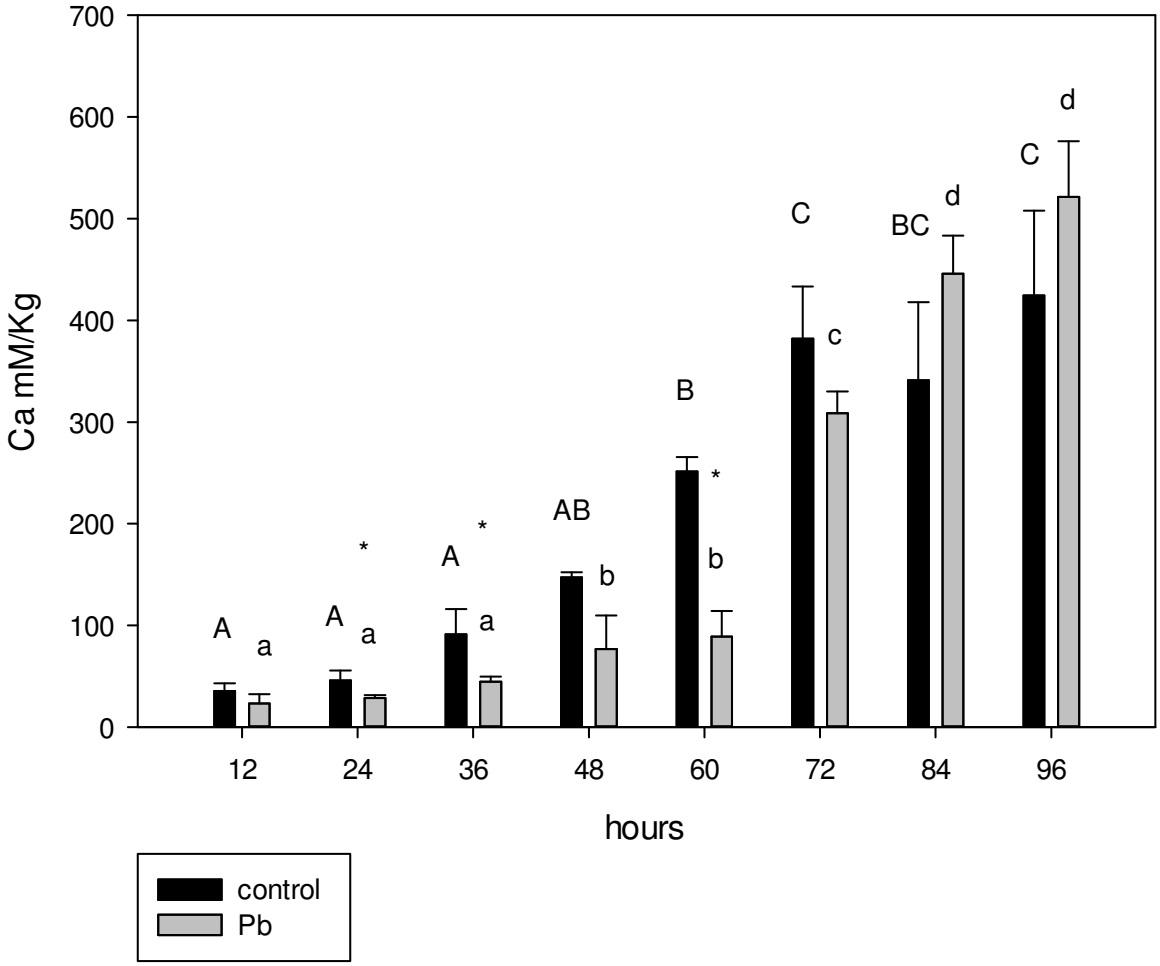
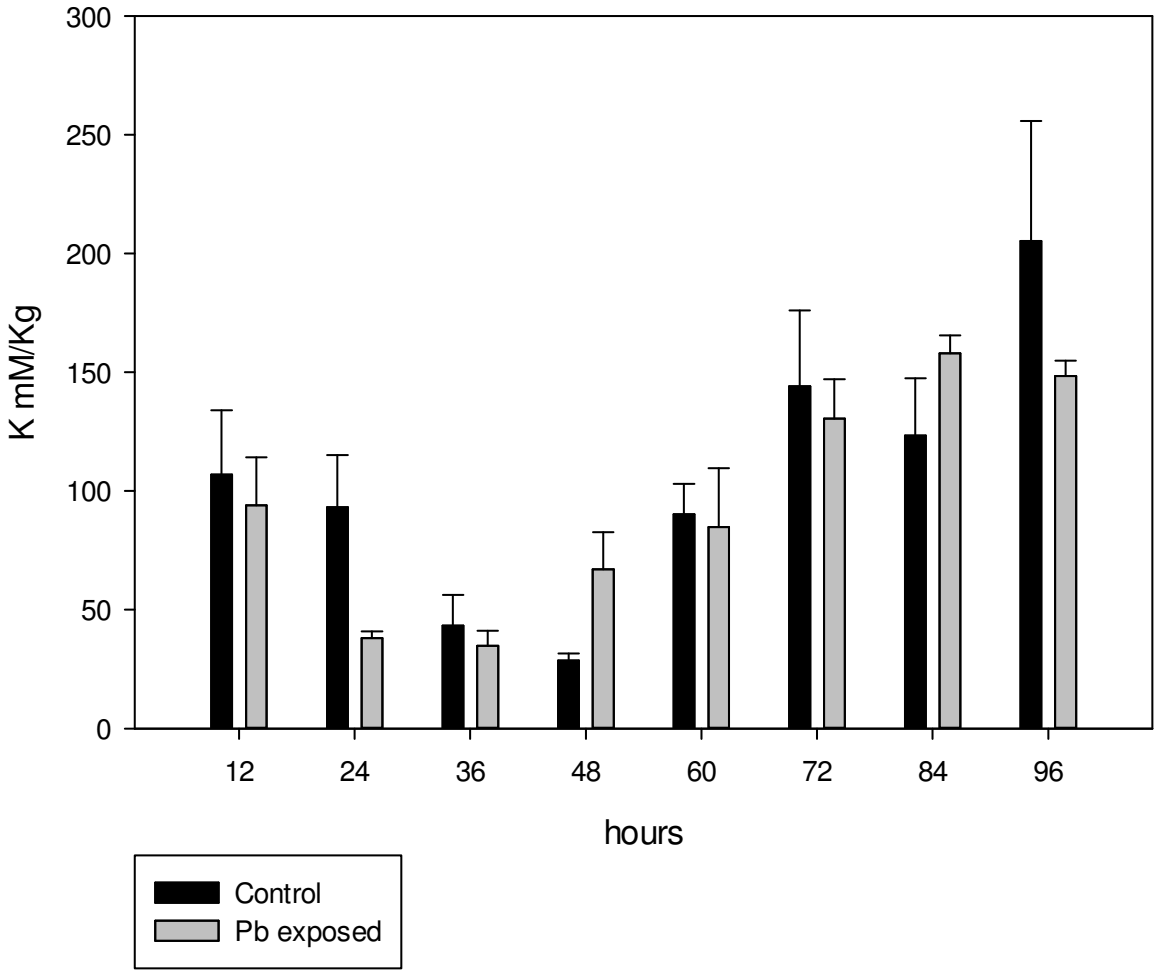

400
300
200
100
0
Mg mM/Kg
*
*
*
12
24
36
48
60
72
84
96
hours
control
Zn exposed

Figure 3-11 Whole body ion levels in larvae exposed to Pb (60 µg/L) over 96 hours of development **a)** Calcium **b)** Potassium **c)** Sodium **d)** Magnesium**.** An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's t-test ($P<0.05$). Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Letters of different cases indicate comparisons within treatments; upper case letters represent comparison between controls and lower case letters represent comparison between treatments. Values are means ± SEM ($N = 5$).

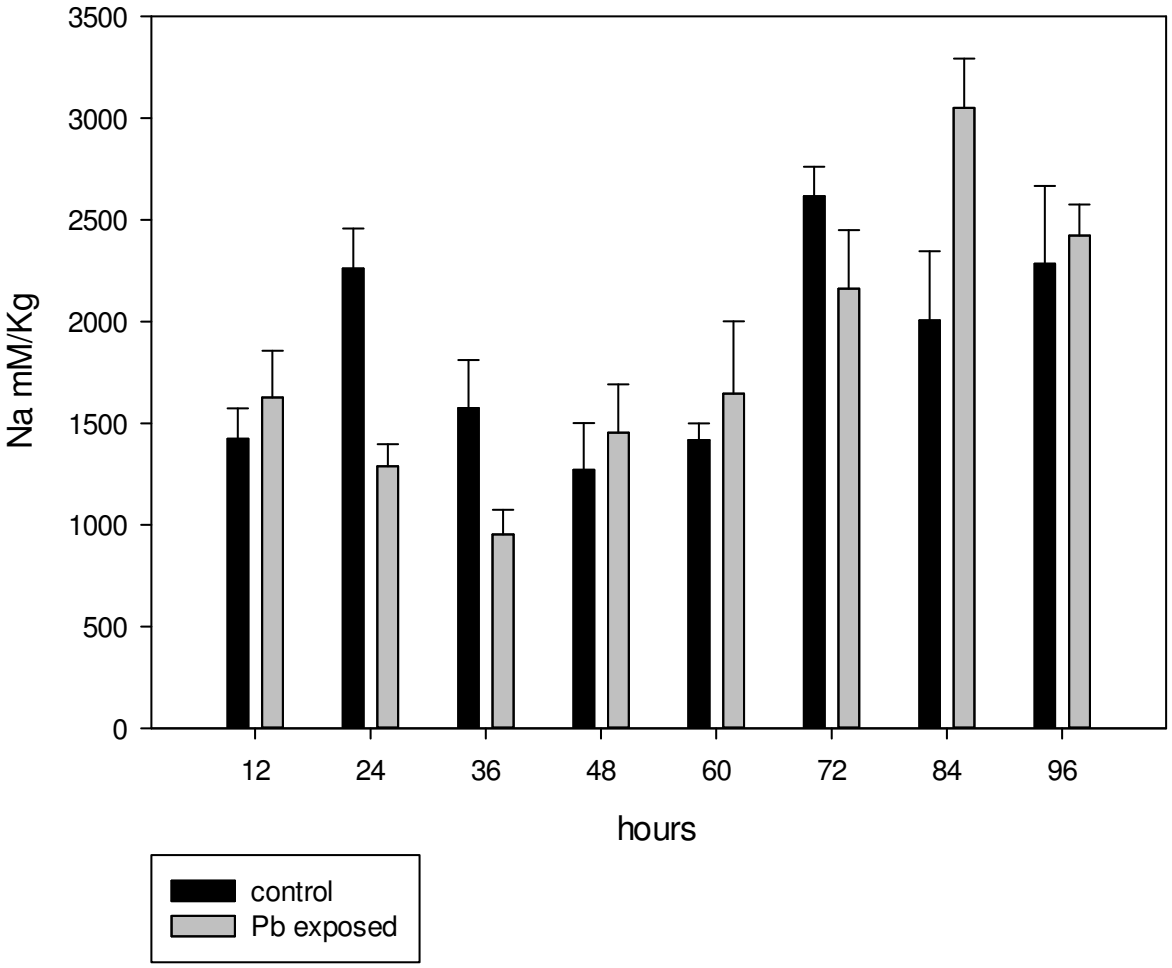
a


700
600
500
400
300
200
100
0
Ca mM/Kg
12
24
36
48
60
72
84
96
hours
A
a
A
a
A
a
AB
b
B
b
C
c
BC
d
C
d
*
*
*
*
control
Pb

b


300
250
200
150
100
50
0
K mM/Kg
12
24
36
48
60
72
84
96
hours
Control
Pb exposed

c


3500
3000
2500
2000
1500
1000
500
0
Na mM/Kg
12
24
36
48
60
72
84
96
hours
control
Pb exposed

d

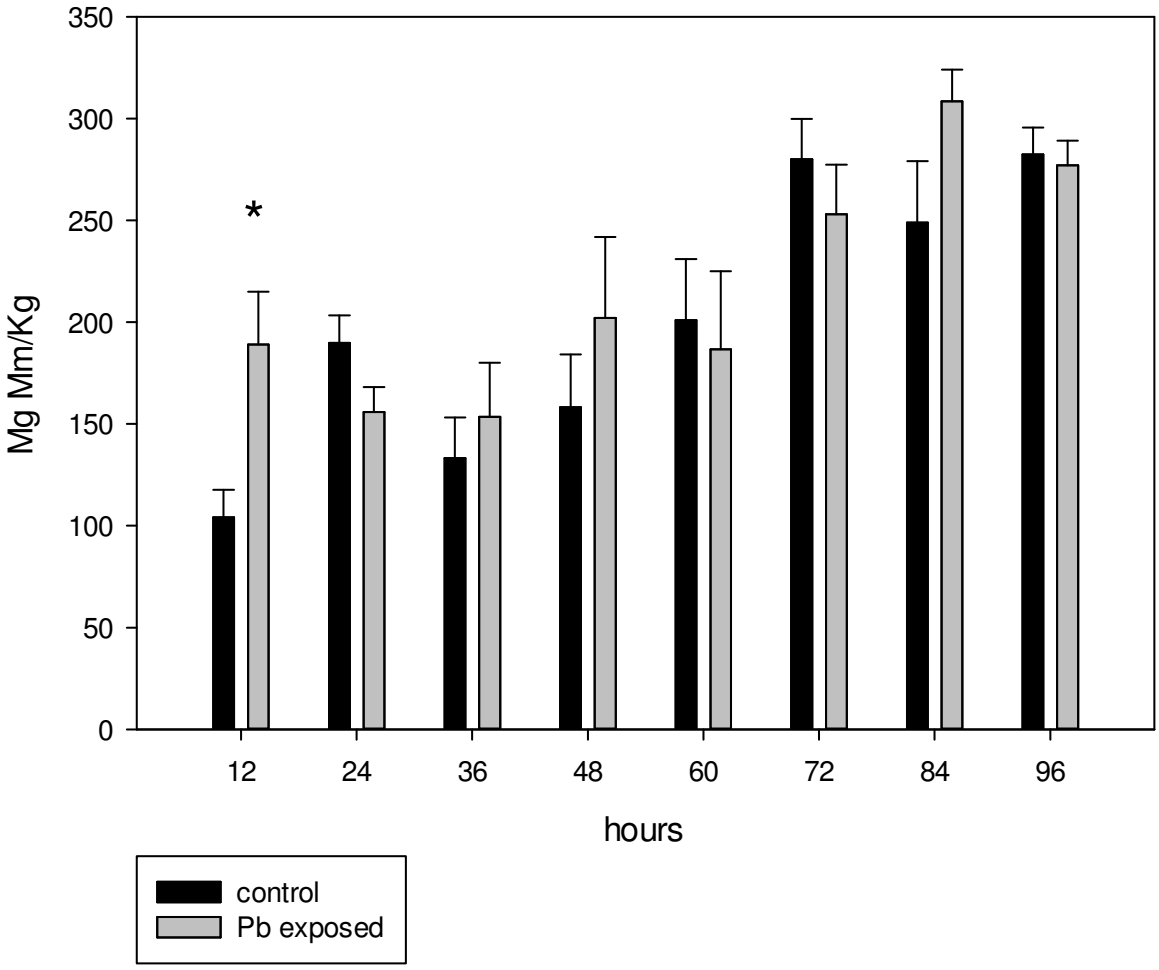
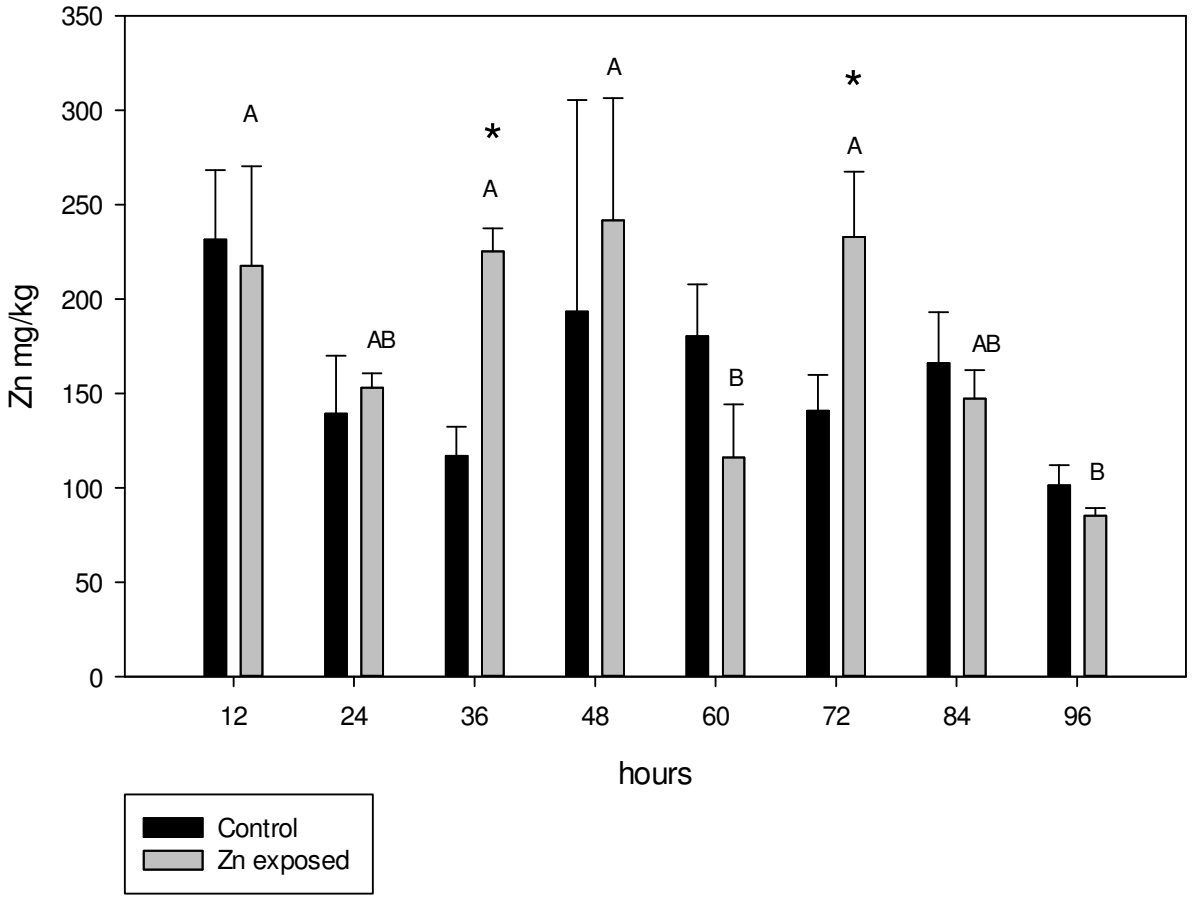
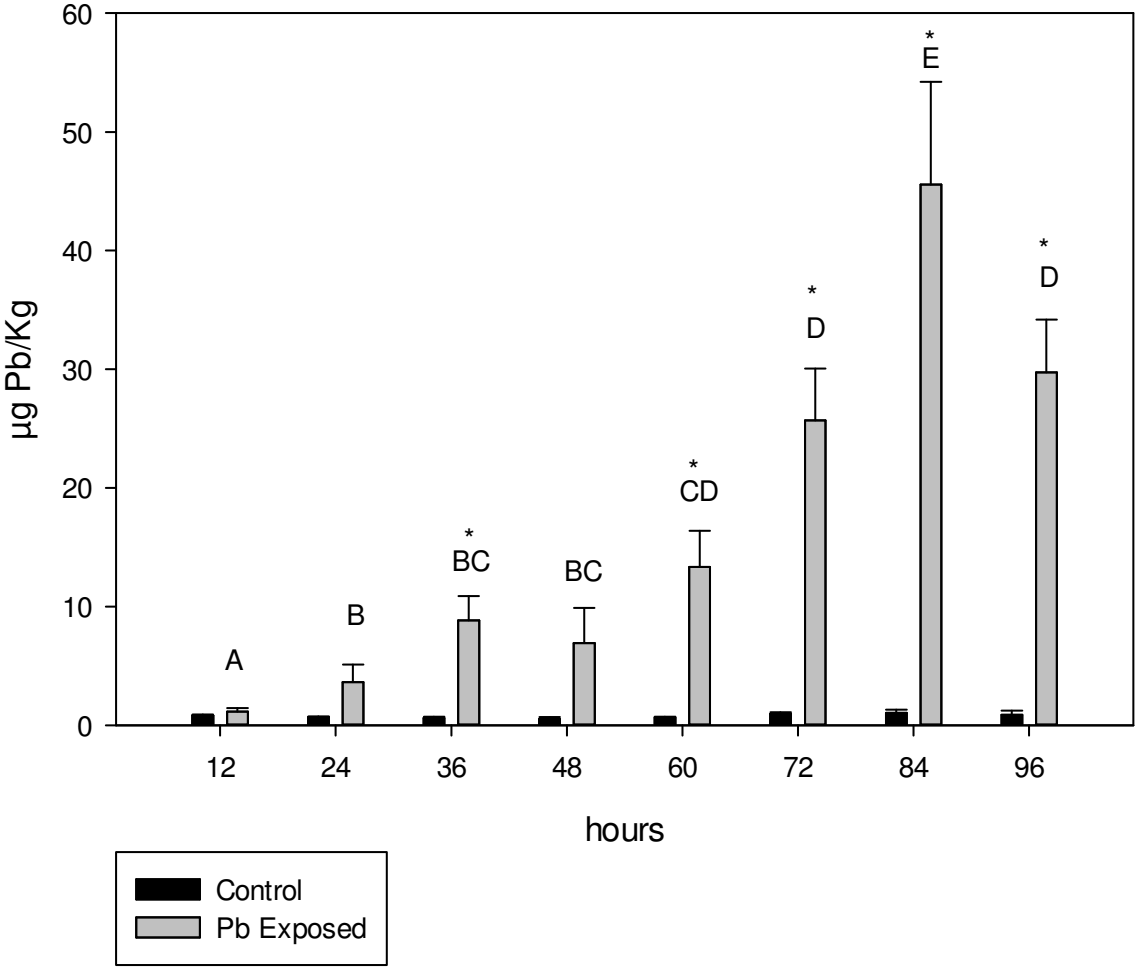

350
300
250
200
150
100
50
0
Mg Mm/Kg
*
12
24
36
48
60
72
84
96
hours
control
Pb exposed

Figure 3-12 Whole body metal accumulation over 96 hours of development **a)** Zn exposed (139 µg/L) **b)** Pb exposed (60 µg/L). An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's t-test ($P<0.05$). Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Letters of different cases indicate comparisons within treatments; upper case letters represent comparison between controls and lower case letters represent comparison between treatments Values are means ± SEM ($N = 5$).

a


350
300
250
200
150
100
50
0
Zn mg/kg
A
AB
*
A
A
B
*
A
AB
B
12
24
36
48
60
72
84
96
hours
Control
Zn exposed

b


60
50
40
30
20
10
0
µg Pb/Kg
A
B
*
BC
BC
*
CD
*
D
*
E
*
D
12
24
36
48
60
72
84
96
hours
Control
Pb Exposed

CHAPTER 4: THE EFFECT OF CU AND NI ON THE EMBRYONIC AND LARVAL STAGES OF DEVELOPMENT

Abstract

Considering the paucity of information on the toxicity of Cu and Ni in the marine environment, the aim of our research was to generate site-specific data on the mechanisms of toxicity of Cu and Ni in the marine environment. A sensitive test organism (*S. purpuratus*) at its most vulnerable life stage (i.e. the embryonic and larval stages) was utilized to study Cu and Ni toxicity during chronic exposure (Cu 6 µg/L and Ni 47 µg/L) as well acute challenges (Cu: 10, 20 and 40 µg/L and Ni: 75, 150 and 300 µg/L). Whole body ions, Ca uptake as well as Ca ATPase activity were the chosen biological endpoints measured every 12 h over the first 84 h of development.

Our research showed that an important mechanism of toxic action of Cu and Ni is ionoregulatory disruption. Most noteworthy was a significant disturbance of Ca homeostasis evident from an inhibition of unidirectional Ca uptake rates and whole body Ca accumulation, as well as by inhibition of Ca ATPase activity intermittently over 96 h of development. Lack of effects after acute metal exposure demonstrates that this inhibition of Ca uptake is not due to direct competitive effects of the metals. At various stages over development, Cu and Ni exposed larvae displayed lower levels of K. This often corresponded with higher levels of Na in these larvae. Higher levels of Mg during initial stages of development in Cu exposed larvae were also observed.

Notably, much of the effects outlined in our research occur during initial stages of development but are reversed by 72 h or 84 h. We therefore propose that it is of value to study the effects of contaminants periodically over development, prior to the traditional endpoint of 72 h.

Introduction

Wide-scale metal contamination of aquatic ecosystems as a consequence of heightened industrial activity over the past century is a major environmental problem (Lander and Reuther, 2004). Cu and Ni are two metals, which have experienced increases in environmental levels as a result of this industrial productivity boom (Wood, 2012).

Cu is primarily used in the production of wire, other electrical products and pipes used in plumbing and its global production has increased exponentially over the past 100 years (Wood, 2012). Regrettably, increased production has resulted in higher amounts of Cu entering aquatic ecosystems through industrial effluent, which has significant impacts on resident biota (Newell, 1997). As an essential trace element, Cu is found in many key biological molecules such as hormones, vitamins, many enzymes and nucleoprotein complexes (Philip, 1977). Due to its importance in several biological processes, Cu levels in organisms are strictly regulated. However, higher levels of Cu introduced to animals through environmental contamination may prove to be toxic and result in Cu acting as a biocide (Warnau et al, 1996). The impact of Cu on the aquatic system is a grave concern as fish, crustaceans and algae exhibit 10 to 1000 times greater sensitivity to Cu than

mammals (Wright and Welbourn, 2002). Cu is known to render its toxic action through altering the activity of membrane-located carrier proteins or ATPases (Li et al., 1996) as well as carbonic anhydrase (Zimmer et al., 2012). This results in a disruption of ionic balances.

Ni is another metal in high demand due to its utilization in the production of stainless steel (Badoo, 2008). Ni is a known Ca homeostasis disruptor, acting antagonistically against Ca and blocking several different types of Ca channels (Lee et al., 1999). Additionally Ca has been observed to be protective against Ni exposure in rainbow trout (*O. mykiss)*, fathead minnows (*P. promelas)* and the water flea (*D. pulex)* (McFarlane and Gilly, 1998; Todorovic and Lingle, 1998 and Deleebeeck et al., 2007). Ni acts as an antagonist to Mg in many mammals, birds, bacteria and fungi (Eisler, 1998). Although, the exact mechanisms of Ni transport in cells are not completely known, Ca and Mg channels have been identified as possible routes of entry (Eisler, 1998).

While extensive research exists on the effects of Ni and Cu in freshwater environments (Grosell, 2012; Pyle and Couture, 2012) there is a paucity of information on these metals in the marine environment (Grosell, 2012; Pyle and Couture, 2012). As a consequence of this lack of information, with the exception of B.C. Canada has yet to establish marine water quality criteria for Cu and Ni (Grossell, 2012; Pyle and Couture, 2012). There is need for site-specific toxicity studies to be performed on organisms in seawater, as freshwater data are not necessarily transferable to a marine setting. Water chemistry in particular has a significant influence on the toxicity of metals, as free ions and organic ligands in seawater can form complexes with dissolved metals thus reducing their bioavailability. Also, relative sensitivities of marine organisms may differ to freshwater organisms due to differing biological and chemical make-up (Grosell, 2012). For the present study we will be looking at the effects of Cu and Ni on the very sensitive *S. purpuratus* (purple sea urchin) during its most sensitive life stage ie. the embryonic and larval stages. While there are many studies on the biochemistry of purple sea urchin embryo (Kominami and Takata, 2008) few studies have examined the effects of metal toxicity to the early developmental stages of the urchin (Philips et al., 2003). Estimations of toxicity of metals are based on generalized cell models and therefore our work is important in addressing the void of information in this field. The effects of Cu and Ni exposure on embryos and larvae in the first 84 h of development will be investigated through endpoints such as Ca uptake, CaATPase activity and ion and metal accumulation. The overarching goal of this research is to elucidate the mechanisms of toxicity of Cu and Ni to a sensitive marine organism, in hopes that this will aid in the development of marine water quality criteria.

Materials and methods

The same methods outlined in Chapter 3 were used to study the chosen biological endpoints of Ca uptake rates, ion accumulation, Ca ATPase activity and weights of larvae chronically exposed to Cu and Ni over development. Ca uptake rates were measured both in acute and chronic exposures of Ni and Cu. All endpoints were measured every 12 h over the first 84 h of development. Cu and Ni exposures were run simultaneously and

share the same control. Ni and Cu concentrations in exposure water (metal precipitated from sea water through method outlined in chapter 3) were determined using graphite furnace atomic absorption spectroscopy (220, Varian Palo Alto, CA, USA).

Nominal concentrations of Cu and Ni for chronic exposure were 3 µg/L and 75 µg/L respectively. These represent approximately 30% of the EC50 for the marine bivalve *Mytilus trossolus* (Nadella et al., 2009) and were deemed acceptable as a sublethal exposure based on evidence of similarities in of bivalve and echinoderm larval sensitivities to metals (Ringwood, 1992). For the acute exposure, concentrations were chosen to be the EC50, 2x the EC50 and 4x the EC50 for Cu and Ni in *M. trossolus* (Nadella et al., 2009). Therefore the actual nominal concentrations tested were 10, 20 and 40 µg/L for Cu and 75, 150 and 300 µg/L for Ni.

Results

Metal exposures

In the first chronic exposure larvae were exposed to either Cu or Ni to measure Ca uptake, ions and metal accumulation in Cu and Ni exposed larvae. The same control set was used for both metals. Cu in this test was measured to be 6 µg/L (nominal value of 3 µg/L) with background levels of 2 µg/L and Ni was measured to be 47 µg/L (nominal value of 75µg/L) with background levels of 7 µg/L.

In the second chronic exposure larvae were exposed to Cu and Ni to measure Ca ATPase in all exposed larvae. The same control set was used for both metals. Cu was measured to be 17 µg/L (nominal value of 3 µg/L) with background levels of 5.10 µg/L. Ni was measured to be 33.78 µg/L (nominal value of 75 µg/L) with background levels of 0.77 µg/L.

Inhibition of ^{45}Ca *uptake*

No inhibition of Ca uptake was observed in the acute challenge of either metal (Figure 4-1a and 4-2a) at any time during the development period.

Inhibition of Ca uptake by chronic metal exposure was observed intermittently over 96 h of development in both metal exposures (Figure 4-1b and 4-2b). Chronic Cu exposure inhibited Ca uptake at the mesenchyme blastula stage (24 hrs), at the early gastrula stage, at gastrulation as well as at 80 hours during the pluteus larvae stage (Figure 4-1b).

Ca uptake in chronically Ni exposed larvae was inhibited at the gastrulation stage as well as at 80 hours when the larvae was in the pluteus larvae stage (Figure 4-2b).

Whole body ion content

Cu had a significant impact on whole body Ca levels in exposed larvae, resulting in lower Ca levels at 24, 36, 60 and 72 h. However, Ca levels recovered to control amounts by 84 hours (Figure 4-3a). Ni exposure also resulted in Ca levels that were significantly lower than controls for the first 60 h of development, after which they returned to control levels (Figure 4-4a).

K levels were significantly lower in Cu exposed larvae at 36 h and 48 h and in Ni exposed larvae at 24 h, 36 h and 48 h, but also returned to normal levels thereafter (Figure 4-3b and 4-4b).

Na levels were higher in Cu exposed larvae at 12 h, 36 h, 48 h and 84 h (Figure 4-3c). Ni exposed larvae also experienced higher Na levels at 36 h, 48 h, 60 h and 84 h (Figure 4-4c).

Similar to Zn and Pb in chapter 3, chronic Cu exposure resulted in increased Mg levels during early stages of development (12 h and 36 h), but Mg concentrations returned to control levels for the remainder of the exposure (Figure 4-3d). Mg levels in Ni exposed larvae were not significantly different from controls at any stage of development (Figure 4-4d).

Ca ATPase activity

Ca ATPase activity in Cu exposed larvae did not differ from control levels at 12 h but was significantly inhibited at 24 h, 36 h and 48 h, after which activity returned to control levels (Figure 4-5a).

Ni exposure had a great impact on Ca ATPase activity for most of the 96 h development period. Significant inhibition of Ca ATPase activity was observed at 12 h, 24 h, 36 h and 48 h of development (Figure 4-5b).

Metal accumulation

Whole body Cu burden accumulated to a significantly higher level than controls at 24 h, in Cu exposed larvae, but that was the only time point over development displaying significant accumulation (Figure 4-6a).

Interestingly, whole body Ni burden was significantly lower in exposed larvae as compared to controls at 48 h, but was not significantly different than controls at any other time point over the 84 h (Figure 4-6b).

Larval weights

Larval weights of Cu and Ni exposed larvae did not differ in comparison to controls over 96 h of development (Figure 4-7a and 4-7b).

Discussion

Analysis of various biomarkers over embryonic development determined that Cu and Ni render their toxic action through ionoregulatory disruption. Toxic effects of Cu and Ni exposure were particularly detrimental to Ca homeostasis.

While controls in this chapter follow the same pattern as the controls in chapter 3 there are differences in the temporal scale of events. For example Ca uptake follows the same pattern in the controls for both chapters, however Ca uptake patterns in Cu and Ni controls appears at later time points than they do in the controls of the preceding Pb and Zn series. This can be attributed to natural biological variability caused by various factors including the second series being run later in the reproductive season as well as gravid

adults spawned for the tests, being obtained later in the summer when the weather was warmer. Toxic effects observed in this chapter however, are more dependent on developmental landmarks rather than time.

Inhibition of Ca uptake over 96 hours of development

Inhibition of Ca uptake was not observed during acute exposure of larvae to metals (Figure 4-1a and 4-2a), meaning that there were no direct competitive effects of the metal on Ca uptake.

Larvae chronically exposed to Cu and Ni however, exhibited intermittent inhibition of Ca uptake over 84 h development. Greatest inhibition of Ca uptake was observed during times when Ca uptake rates were highest over development. This was during the blastula (24 h and 36 h), gastrulation (60 h) and pluteus larval (84 h) stages in Cu exposed larvae (Figure 4-1b) and during the gastrulation (60 h) and pluteus larval (84 h) stages in Ni exposed larvae (Figure 4-2b). Sub optimal function of Ca voltage-gated channels might be the reason why exposed larvae were unable to meet the increased demands for Ca transport during these stages. Extracellular transition metals such as Cu and Ni are known to inhibit voltage-gated Ca channels through binding to negative sites close to the gating apparatus. This causes channel opening to be slowed, as a more positive potential for activation is required (Gilly and Armstong, 1982). Impairment of these channels by Cu and Ni may not be dose dependant however, as Büsselberg et al. (1992) showed that sometimes lower doses of extracellular transition metals resulted in greater inhibition.

Inhibition of Ca uptake was most pronounced at the gastrulation stage in both Cu and Ni chronic exposures (Figure 4-1b and 4-2b). Similar to these findings, Pb and Zn exposure also had the greatest effect on Ca uptake at the gastrulation stage, as outlined in our work in chapter 3. The gastrulation stage is known to be an especially critical and vulnerable stage of development as abnormalities at this phase often result in complications in later development of the skeleton (Yaroslavetseva and Sergeeva, 2002). Larvae are particularly susceptible to metal toxicity during gastrulation as maternal sources of metallothioneins are exhausted by this stage (Warnau, 1996). Metallothioneins are a family of cysteine rich proteins, characterized by a high affinity for metals. They have a protective function in that these proteins bind metals, thus reducing their bioavailability (Warnau, 1996). A depletion of these proteins at the gastrulation stage therefore leaves embryos increasingly vulnerable to metal toxicity until increased synthesis of their own metallothioneins is initiated (Warnau, 1996). It is therefore understandable that this stage would display greater effects of toxicity relative to the other stages of development. Newly synthesized metallothioneins may be more effective in their protective role against metals, as they have not been previously exposed to metals as maternal metallothioneins might have been. Evidence for this is seen in a return to normal of Ca, K as well Ca ATPase levels in Cu and Ni exposed larvae in stages after gastrulation as discussed later (Figure 4-3a and b; Figure 4-4 a and b; Figure 4-5 a and b).

Whole body ion and metal content over development

Sublethal toxic effects of Cu and Ni were also apparent from whole body ion levels in metal exposed larvae. Major ions (Mg, Ca, K and Na) measured in Cu and Ni exposed larvae over development, displayed deviances from their normal concentrations, with the exception of Mg in Ni exposed larvae. This indicates that ionoregulatory disruption may be a key mechanism of Cu and Ni toxicity.

Na levels were higher in exposed larvae at various time points over development often at the same time points as when an inhibition of K levels was occurring (Figure 4-3b and c; Figure 4-4b and c). Na/K ATPases function to maintain elevated intracellular K by pumping out 3 Na for every 2K pumped into the cell. High Na content observed at the same time K content is decreased therefore suggests a malfunction of these important proteins. Past studies show an inhibition of Na transport in coinciding with an inhibition of the Na/K ATPase enzyme in freshwater fish (Lauren and McDonald, 1985, 1987a, 1987b) as well as marine invertebrates (Lopes et al., 2011). Whether a similar inhibition of this enzyme occurs in sea urchin larvae can be confirmed through Na/K ATPase analysis in future experiments. Alteration of activity of this enzyme has serious consequences as Na/K ATPase controls membrane effective permeability by transporting ions across the cell membrane, which in turn modulates cell volume and osmotic pressure (Reddy and Philip, 1992). Clearly, an inhibition of this enzyme would not only affect absolute levels of K and Na, but would also indirectly elicit a variety of negative consequences on general cell health.

This affect on Na was not observed in Pb and Zn exposed larvae (chapter 3), however Cu has been specifically implicated in disruption of Na homeostasis in numerous studies (Lopes et al., 2011; Grossell et al., 2004). Interestingly Ni is not a known Na disruptor however effects of Ni on Na regulation were observed in the present study.

Other ions affected by metal exposure were Ca and Mg. Lower levels of Ca were observed in both Cu and Ni exposed larvae during the initial stages of development (Figure 4-3a and 4-4a) and Mg levels were higher than controls during the initial stages of development in Cu exposed larvae (Figure 4-4d). Both of these ions play an important role in spicule formation, which makes disruption of their homeostasis by metals more noteworthy (Raz et al., 2003). Higher levels of Mg in Cu exposed larvae could be a compensatory mechanism employed by the larvae to counter metal induced Ca disruption. Interestingly Ni, a known Mg antagonist (Li et al., 1996) did not have an effect on Mg levels in developing sea urchins.

Lower levels of Ca in exposed larvae indicate a disruption of Ca homeostasis, which may have serious implications on normal sea urchin larval development considering this ion is a major constituent of the spicule (Raz et al., 2003). Interestingly, there was a latent period in-between inhibition of Ca uptake by metal exposure and resulting lower levels of Ca accumulated in the metal exposed larvae. In Cu exposed larvae, inhibition of Ca uptake at 24 hours (Figure 4-1b) resulted in lower larval concentrations of Ca after 36 hours of development (Figure 4-3a). A return to normal Ca uptake rates at 48 hours (Figure 4-1b) coincided with normal Ca concentrations at 48 hours (Figure 4-3a), after which inhibition of Ca uptake at 60 and 72 hours (Figure 4-1b)

coincided with lower Ca concentrations at 60 and 72 hours (Figure 4-1b and 4-3a).

In larvae exposed to Ni, disturbances of whole body Ca concentration did not follow the same pattern as seen for Cu exposure. Lower Ca concentrations in Ni exposed larvae were seen at 12 hours of development until 60 hours (Figure 4-3a), well before inhibition of Ca uptake occurred at 60 and 96 hours (Figure 4-2b). Ca flux measurements in these experiments are unidirectional and therefore the efflux rate of Ca is unknown. Larvae exposed to Ni may have an impaired ability to retain imported Ca resulting in lower levels being accumulated. This would explain lower Ca accumulation in the larvae prior to the appearance of Ca uptake. Metal induced stimulation of Ca release from endocellular stores is not a novel phenomenon and has been observed in numerous studies on marine invertebrates (Viarengo, et al., 1994; Viarengo et al., 1991; Walter et al., 1989)

Ca accumulation returned to normal after the skeletogenic gastrulation stage (approximately 60 h) (Figure 4-4a) in spite of Ca uptake inhibition occurring around this time (Figure 4-2b). This might indicate that once Ca is incorporated into the matrix of the spicule, it is less susceptible to loss through efflux. An approximation of the Ca efflux at each stage of development was determined by calculating the theoretical Ca accumulation based on the influx rate of Ca every 12 h and subtracting the measured Ca content of the larvae every 12 h to indicate how much Ca was lost to efflux over development (data not shown). These calculations showed that there was an approximately 62 % decrease in efflux from 60 h till 84 h, which supports this theory.

Ca ATPase activity

Further indication of a disruption in Ca homeostasis was evident from an inhibition of Ca ATPase by Cu and Ni in chronically exposed larvae (Figure 4.5a and b). (NB. Cu exposure for Ca ATPase measurement in larvae, were measured to be higher than the exposure concentrations for other endpoints in this study. Refer to Results for details). An inhibition of Ca ATPase by metals has been observed before in marine invertebrates in our own research (chapter 3) as well as in past studies (Viarengo et al., 1991; 1993). The inhibition could in part be due to the free radical generation ability of these metals. Much of the toxicity caused by metals is due to oxidative stress from metal-catalyzed free radical production. Reactive oxygen species alter the fatty acid profile of the cell membrane, thus in turn disrupting modulation of membrane-bound ATPases whose activity depends on membrane phospholipids (Reddy and Philip, 1992).

ATPase enzymes are also implicated in the transport of metals across plasma membranes of cells as ATP-dependent facilitated diffusion may mediate their uptake. This can occur either through transfer by proteins intrinsic to the cell membrane with specificity for metal ions, or through receptor mediated-endocytosis (Viarengo et al., 1996). Inhibition of ATPase enzymes has been associated with metals binding with the sulfhydral groups of these enzymes, which alters the transport of ions into cells (Pivovarova and Laerspetz, 1996).

Metal accumulation

In Ni exposed larvae, Ni accumulation was lower in exposed larvae in comparison

to controls at 48 hours of development, but the same as controls otherwise indicating no significant accumulation of the metal (Figure 4-6b). Cu exposed larvae, displayed significant accumulation at 96 hours of development but the larvae displayed some capacity to detoxify the metal, as Cu levels decreased significantly over the exposure period (Figure 4-6a).

Larval weights

Metal accumulation was not reflected by larval weights, which did not differ from controls at any time point over development. Larval weights of exposed larvae were consistently the same as controls all throughout development, which suggests that they are not a sensitive endpoint of toxicity for these two metals.

Return to homeostasis

What is apparent from the Ca uptake flux experiments, Ca ATPase activity measurements and the ion accumulation measurements in larvae over development is that larvae possess some capacity to recover from metal stress. This is exhibited by K, Na, Mg and Ca accumulation as well as Ca uptake rates periodically returning to normal at various time points over the metal exposure. By the end of 72h or 84 h, larvae had regained normal whole body concentrations of Ca, K and Mg in the Cu and Ni exposures (Figure 4-3a, b and d and 4-4a, b and d). This leads us to believe that larvae possess damage-repair mechanisms, which reverse the toxic effects of metal. This however does not exclude the fact that larvae have evidently been injured by metal exposure during key early developmental stages. Although this damage is not apparent from measurements of biomarkers at 72 hours of development, alteration of larval homeostasis in early developmental stages could have implications on the later health of adult sea urchins.

Classic toxicity tests are quantified based on the effects of contaminants displayed at 72 h (ASTM, 1994). Our research suggests that a more accurate way of determining toxicity of sub lethal concentrations of metals is to monitor toxic effects periodically over development. This prevents concentrations of contaminants, which do not elicit a response at 72 h from being included within the No Observed Effect Concentration (NOEC) range, when in reality toxic effects have appeared earlier in the larvae. These symptoms of toxicity occurring early in development are important to take into consideration, even though they are not apparent at 72 h as they may still have implications on later health of the urchin.

Conclusion

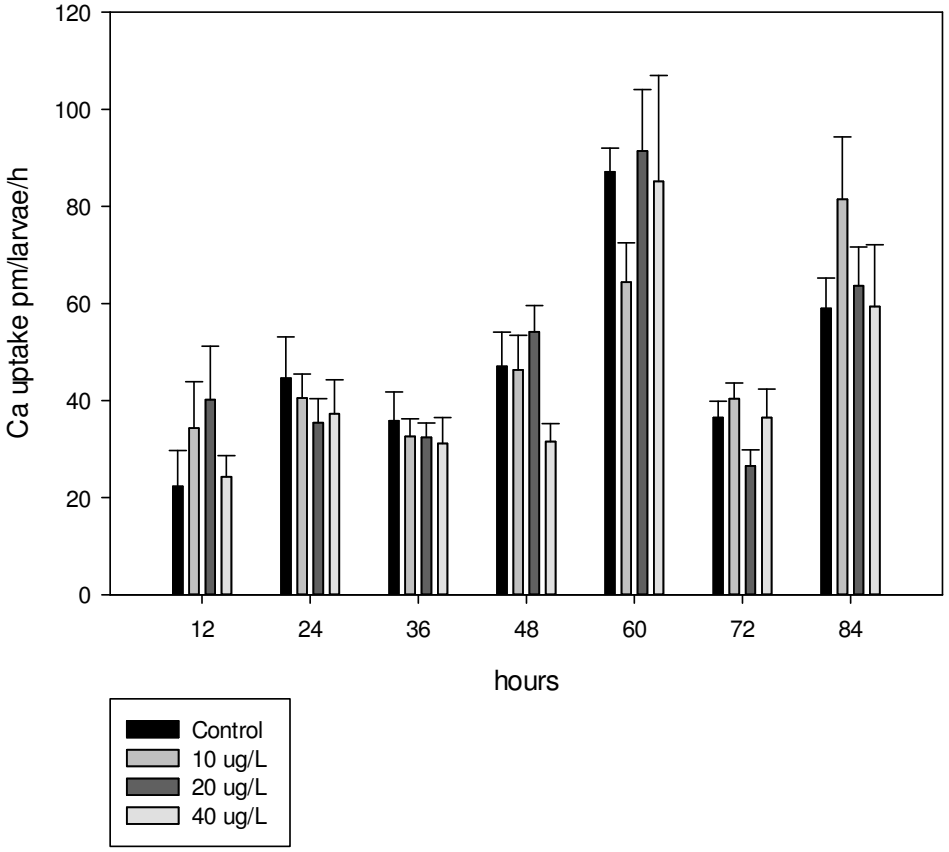
Our research shows that the main mechanism of toxic action of Cu and Ni is ionoregulatory disruption. In particular, a significant disturbance of Ca homeostasis was evident from an inhibition of Ca uptake rates and accumulation as well as Ca ATPase activity. This toxic effect is of notable significance as Ca is vital to sea urchin embryos in its roles in cell division and structural development. A disruption in Ca levels is therefore extremely detrimental to development. Na and K levels were also disrupted by Cu and Ni exposure. This has implications on general cell health as these ions are important in

osmoregulation and maintaining cell volume.

It is interesting to note that much of the effects outlined in our research occur during very early developmental stages but are reversed by 72 h or 84 h of development. We therefore propose that it is of value to study the effects of contaminants periodically over development, prior to the traditional endpoint of 72 h.

Figure 4-1. Ca uptake rates measured every 12 hours over the first 84 hours of larval development in **a)** acutely Cu exposed larvae **b)** chronically Cu (6 ug/L) exposed larvae. An asterisk (*) indicates a significant difference from control levels as determined with a Student's t-test ($P<0.05$). $N = 6$; values are means ± SEM.

a


120
100
80
60
40
20
0
Ca uptake pm/larvae/h
12
24
36
48
60
72
84
hours
Control
10 ug/L
20 ug/L
40 ug/L

b

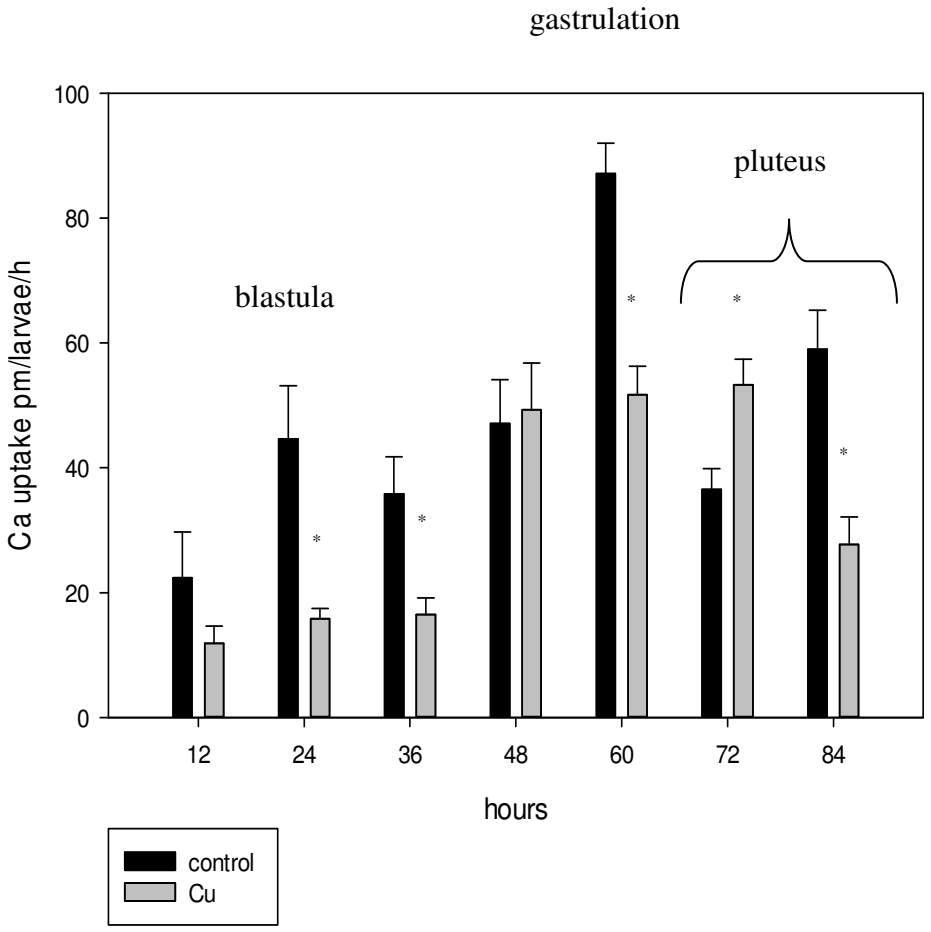
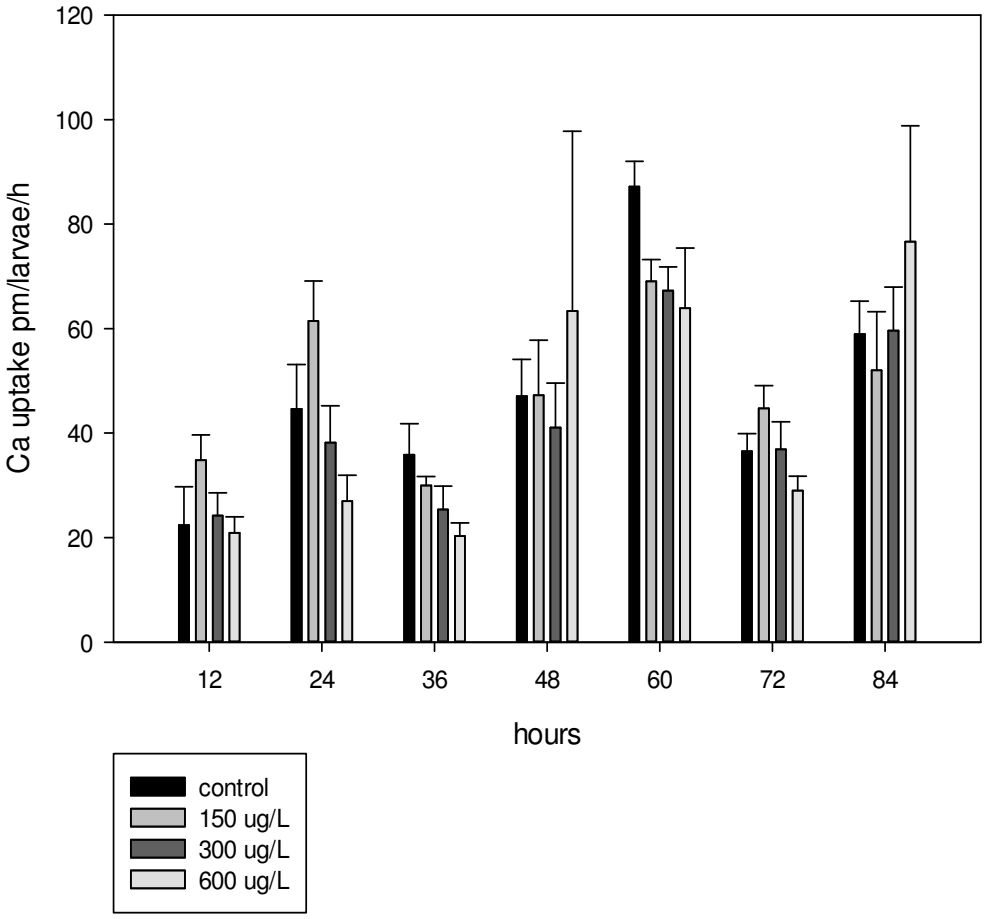

gastrulation
pluteus
blastula
Ca uptake pm/larvae/h
100
80
60
40
20
0
12
24
36
48
60
72
84
hours
control
Cu
*

Figure 4-2. Ca uptake rates measured every 12 hours over the first 84 hours of larval development in **a)** acutely Ni exposed larvae and **b)** chronically Ni exposed (47 ug/L) larvae. An asterisk (*) indicates a significant difference from control levels as determined with a Student's t-test ($P<0.05$). Values are means ± SEM ($N = 6$).

a


120
100
80
60
40
20
0
Ca uptake pm/larvae/h
12
24
36
48
60
72
84
hours
control
150 ug/L
300 ug/L
600 ug/L

b

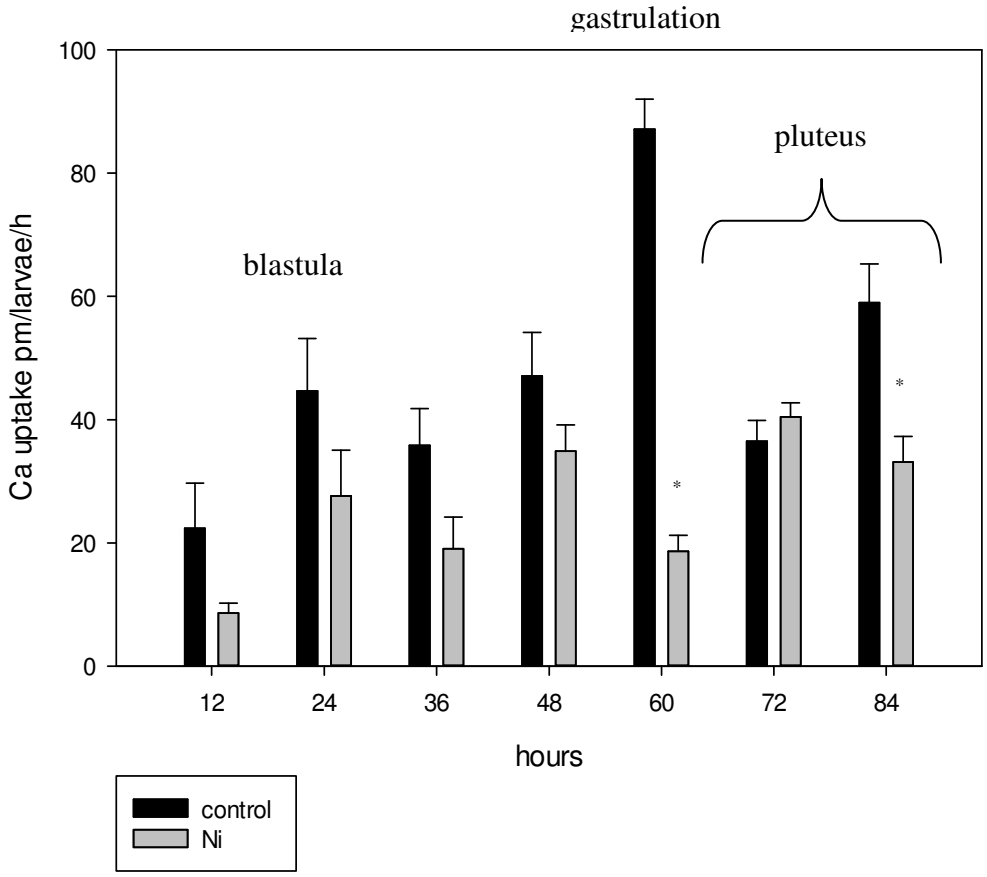
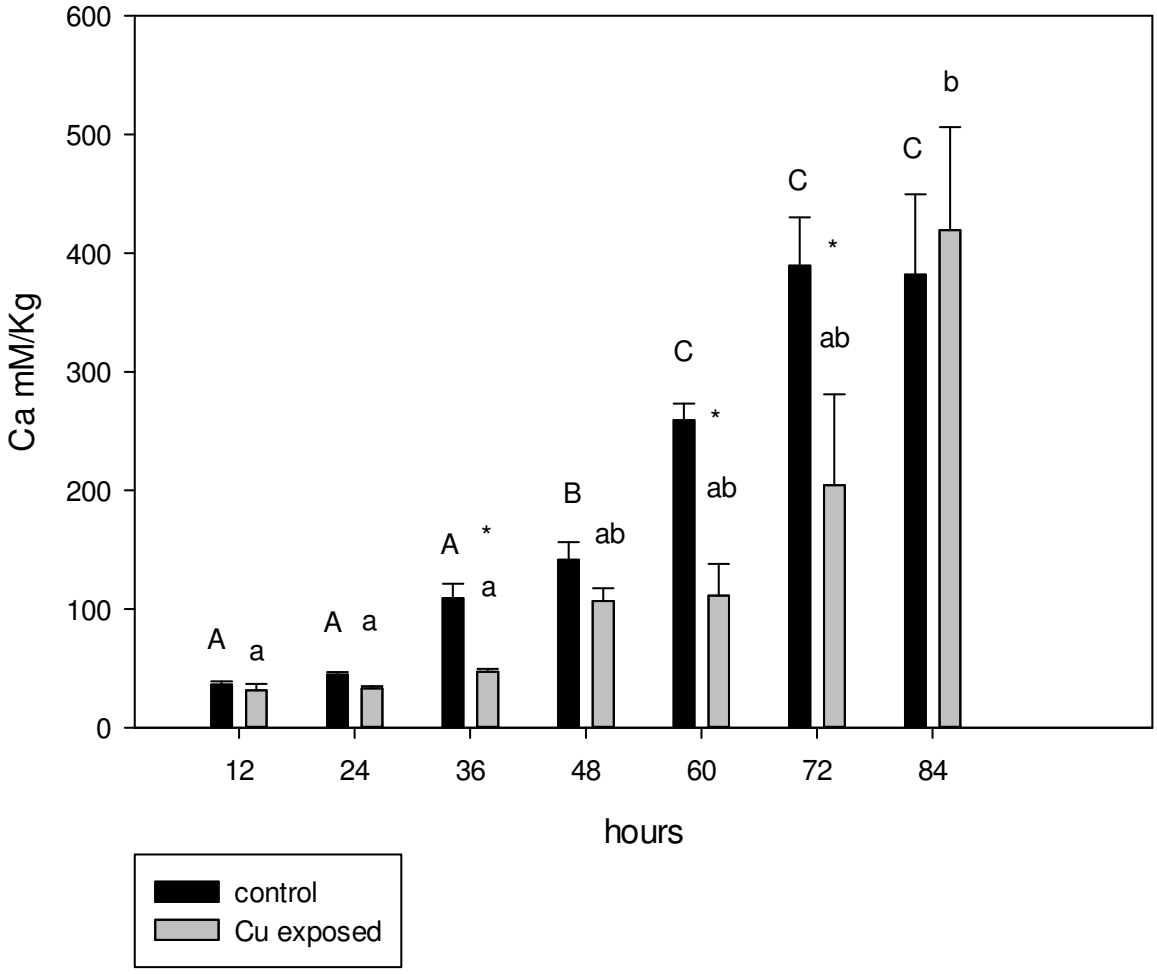
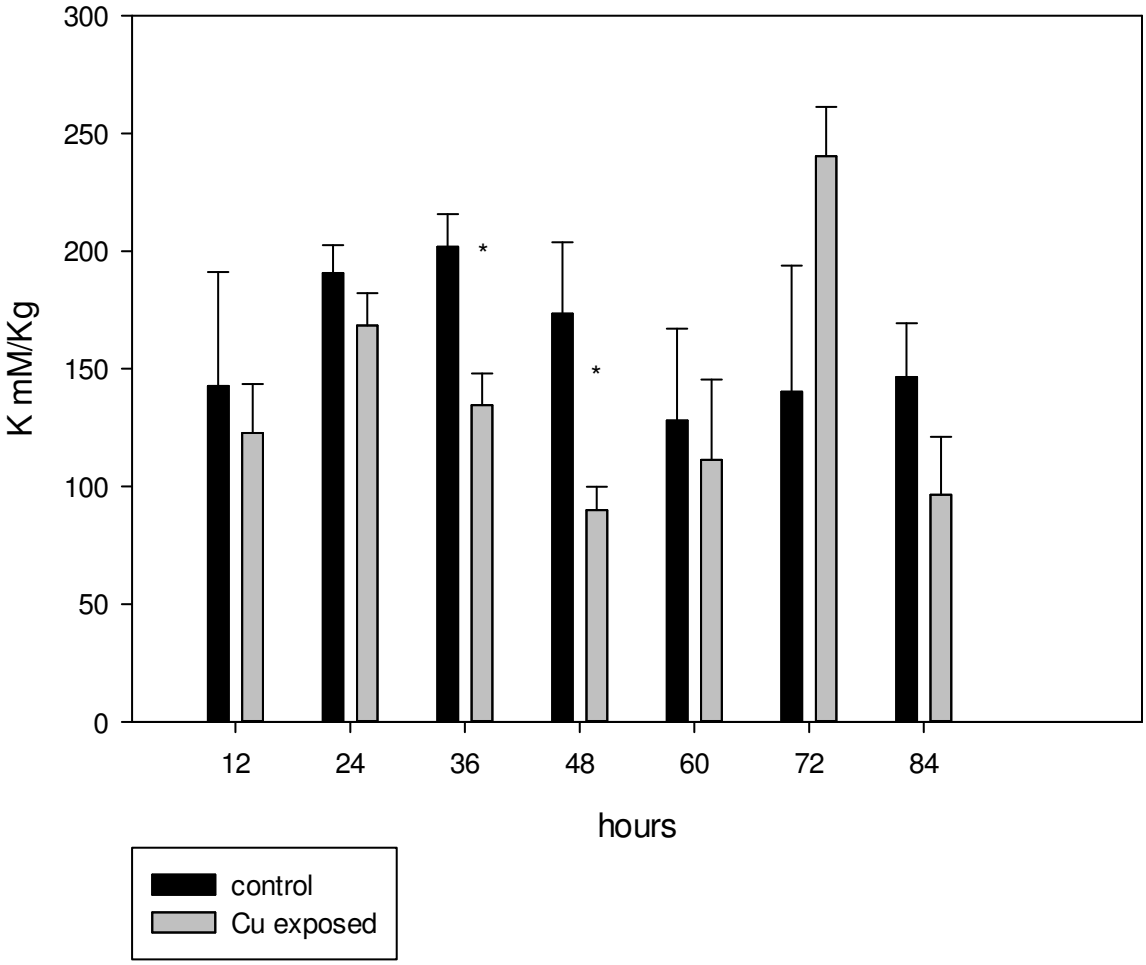

gastrulation
pluteus
blastula
Ca uptake pm/larvae/h
100
80
60
40
20
0
12
24
36
48
60
72
84
hours
control
Ni
*
*

Figure 4-3. Whole body ion levels in larvae exposed to Cu (6 µg/L) measured every 12 hours over the first 84 hours of larval development **a)** Calcium **b)** Potassium **c)** Sodium **d)** Magnesium**.** Controls N=3, exposed larvae. An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's t-test ($P<0.05$). Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Letters of different cases indicate comparisons within treatments; upper case letters represent comparison between controls and lower case letters represent comparison between treatments Values are means ± SEM ($N = 5$).

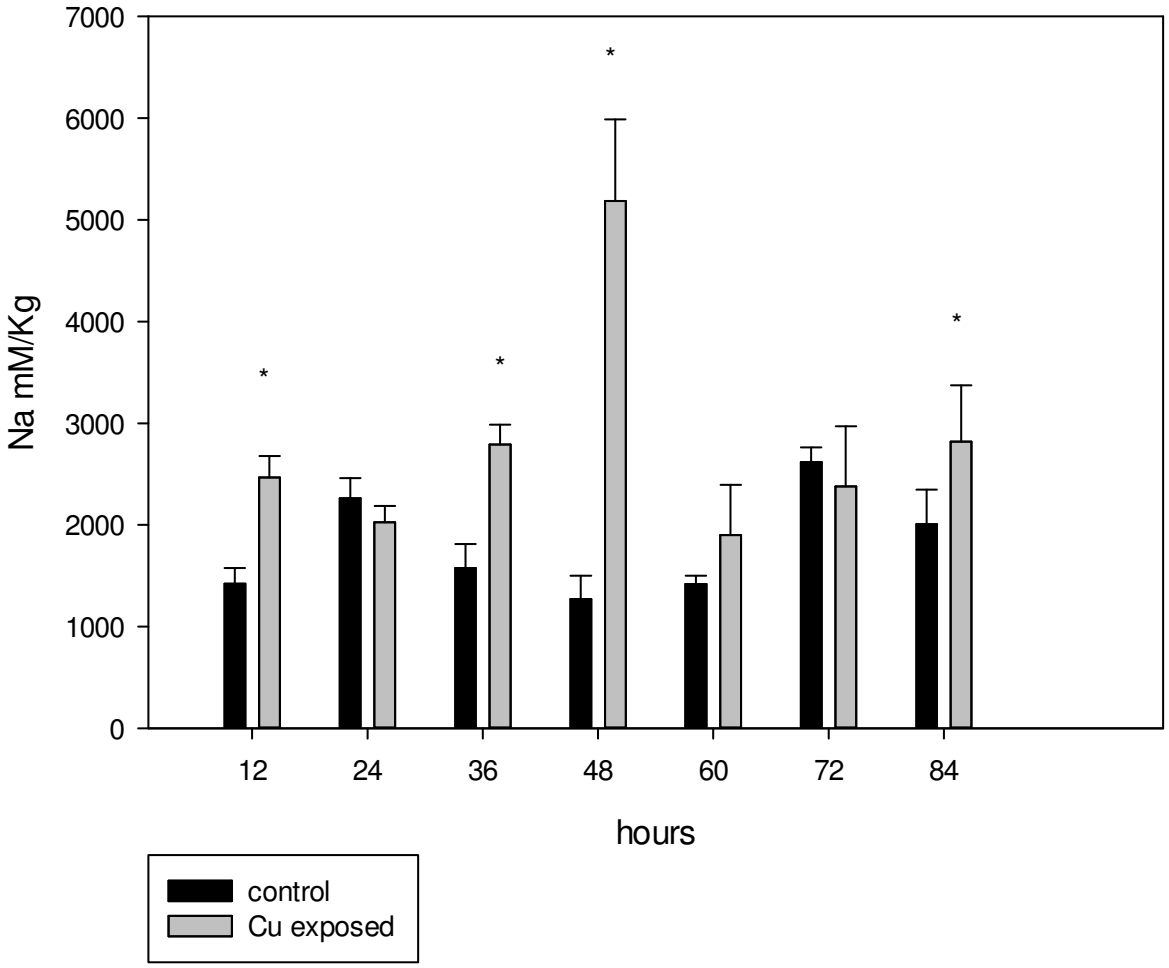
a


600
500
400
300
200
100
0
Ca mM/Kg
A
a
A
a
A
*
a
B
ab
C
*
ab
C
*
ab
C
b
12
24
36
48
60
72
84
hours
control
Cu exposed

b


300
250
200
150
100
50
0
K mM/Kg
*
*
12
24
36
48
60
72
84
hours
control
Cu exposed

c


7000
6000
5000
4000
3000
2000
1000
0
Na mM/Kg
*
*
*
*
12
24
36
48
60
72
84
hours
control
Cu exposed

d

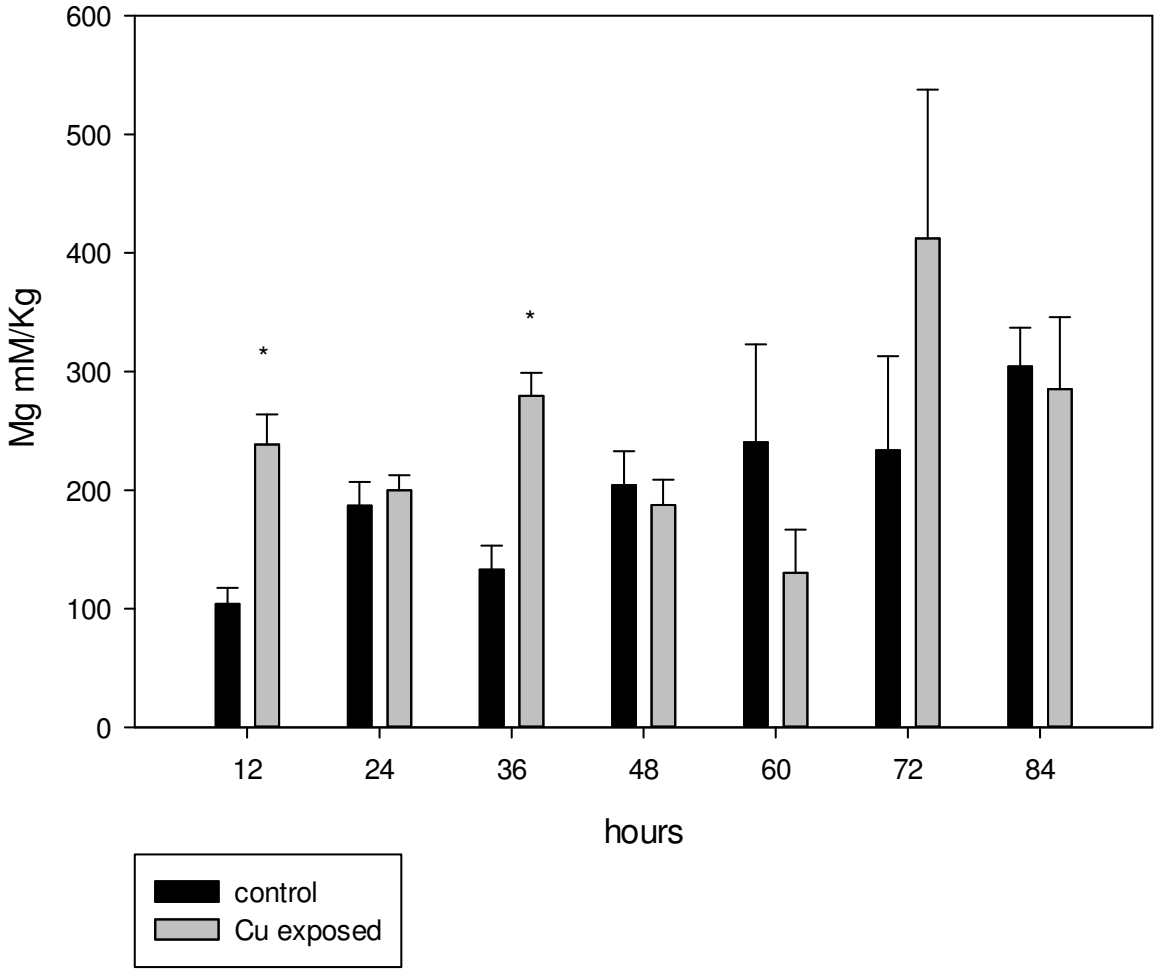
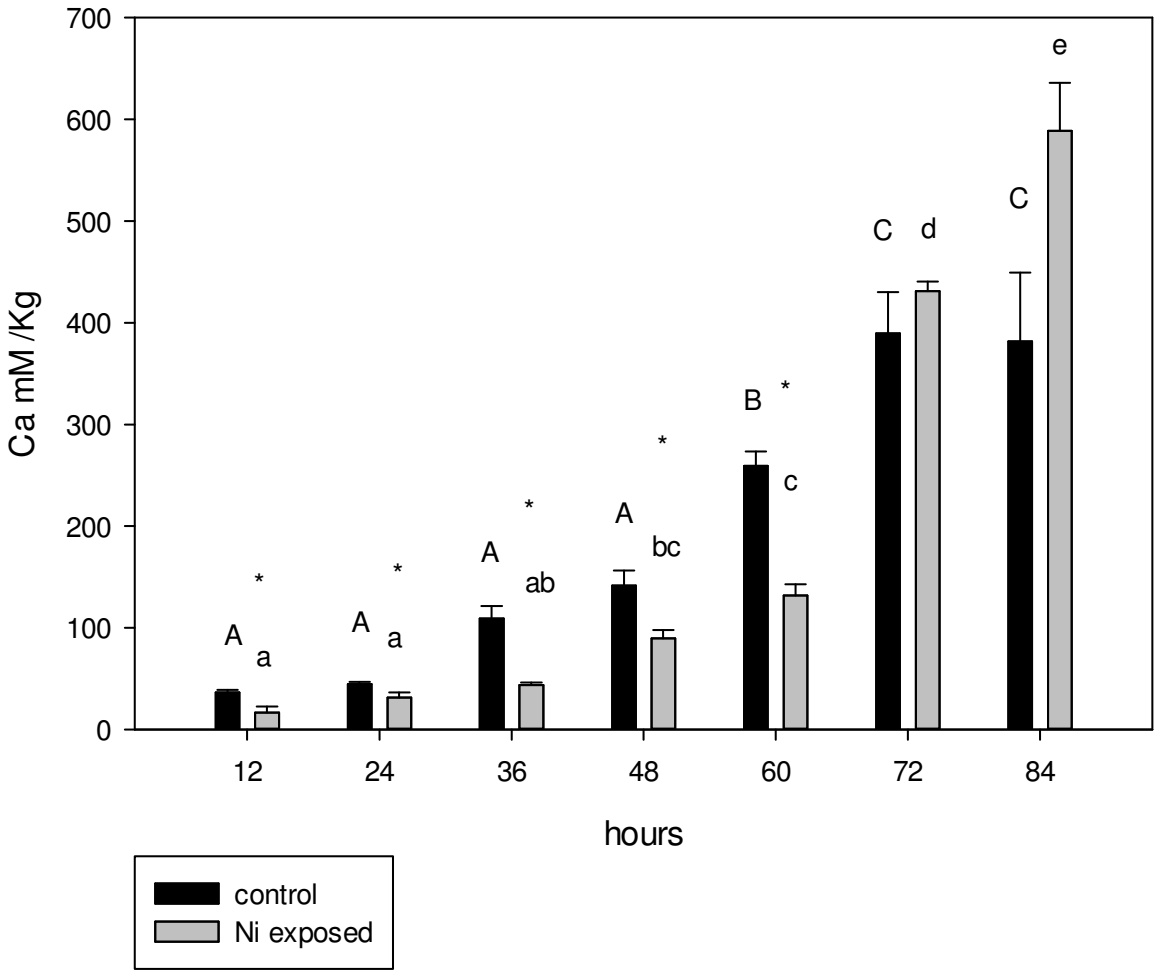
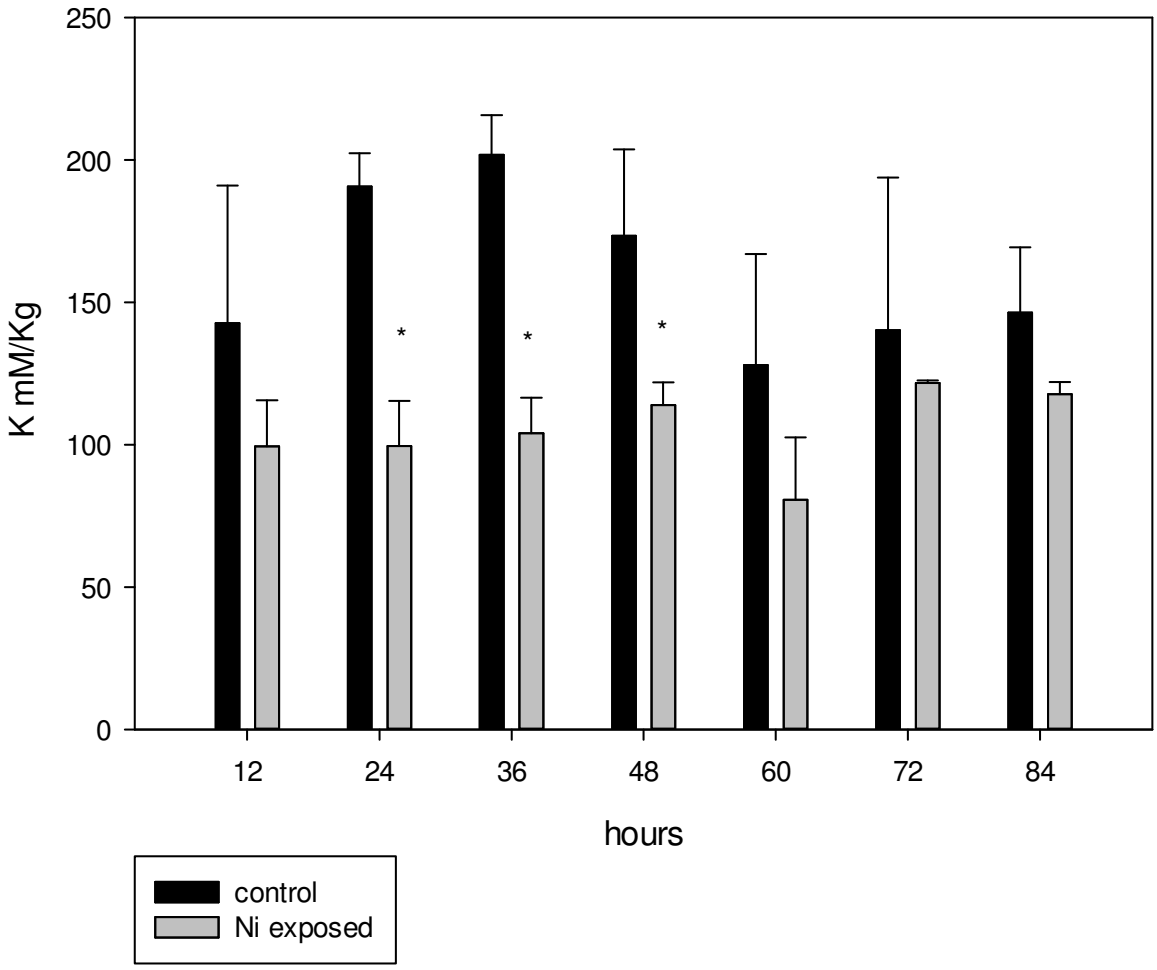

600
500
400
300
200
100
0
Mg mM/Kg
*
*
12
24
36
48
60
72
84
hours
control
Cu exposed

Figure 4-4. Whole body ion levels in larvae exposed to Ni (47 ug/L) measured every 12 hours over the first 84 hours of larval development **a)** Calcium **b)** Potassium **c)** Sodium **d)** Magnesium**.** Controls N=3, exposed larvae. Values are means ± SEM (N = 5).

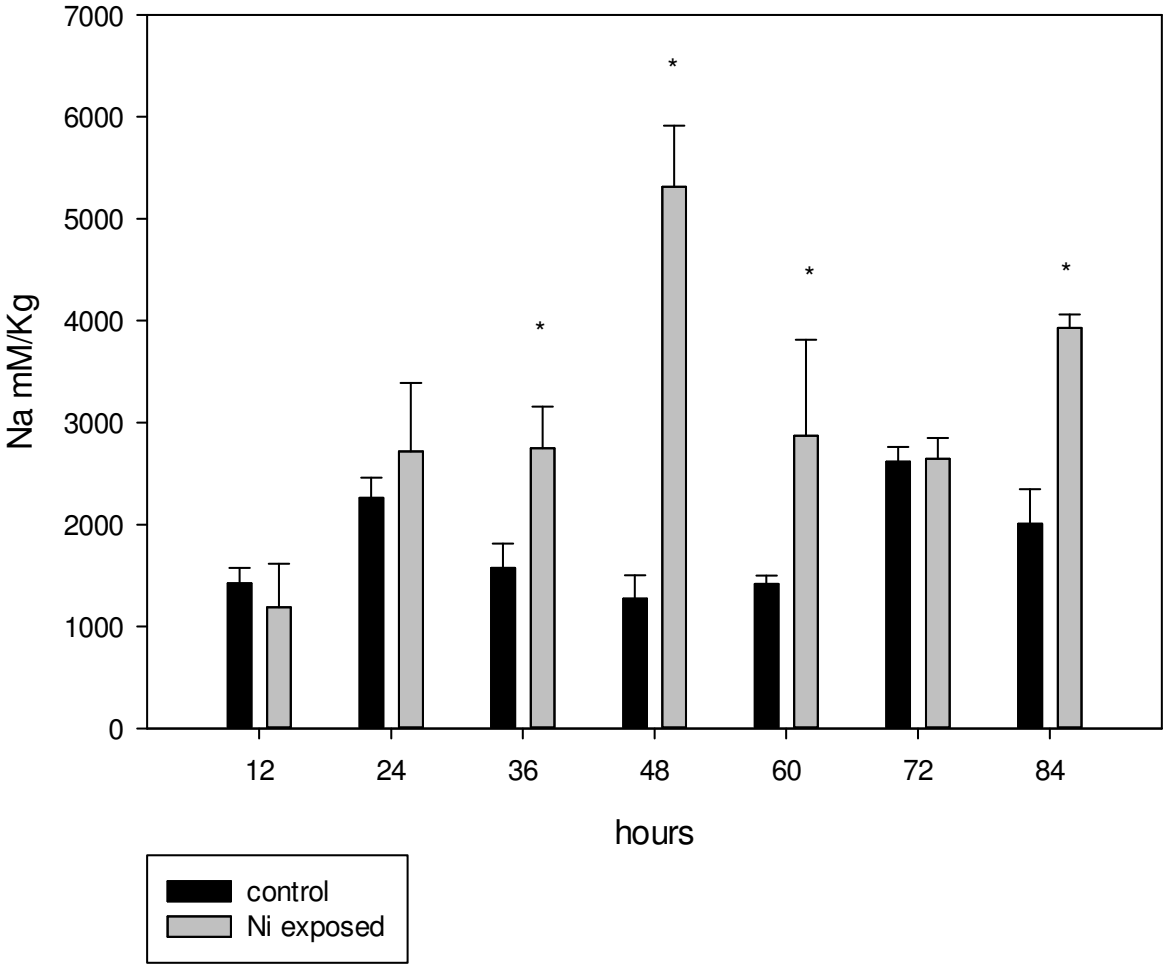
a


700
600
500
400
300
200
100
0
Ca mM /Kg
12
24
36
48
60
72
84
hours
A
a
A
a
A
ab
A
bc
B
c
C
d
C
e
*
control
Ni exposed

b


250
200
150
100
50
0
K mM/Kg
*
*
*
12
24
36
48
60
72
84
hours
control
Ni exposed

c


7000
6000
5000
4000
3000
2000
1000
0
Na mM/Kg
*
*
*
*
12
24
36
48
60
72
84
hours
control
Ni exposed

d

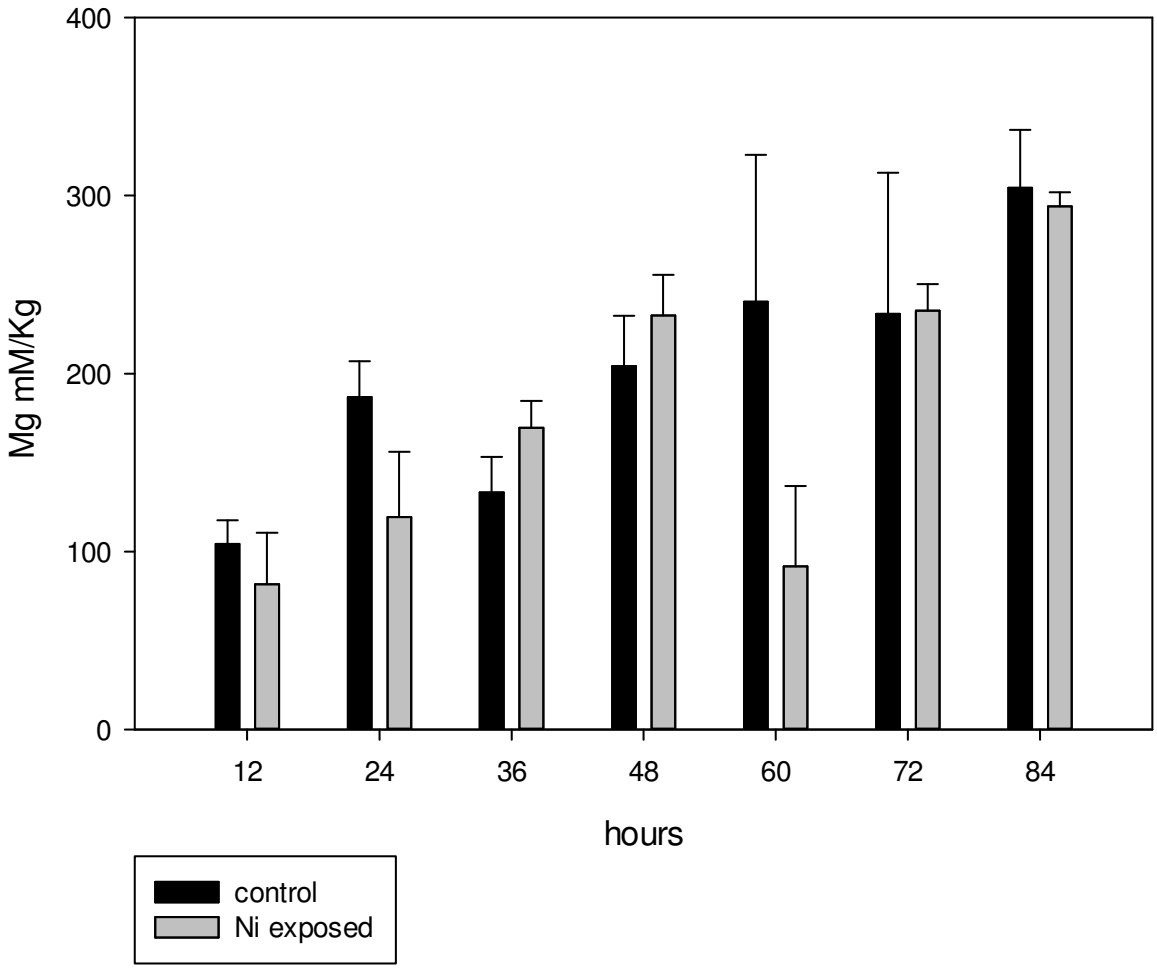
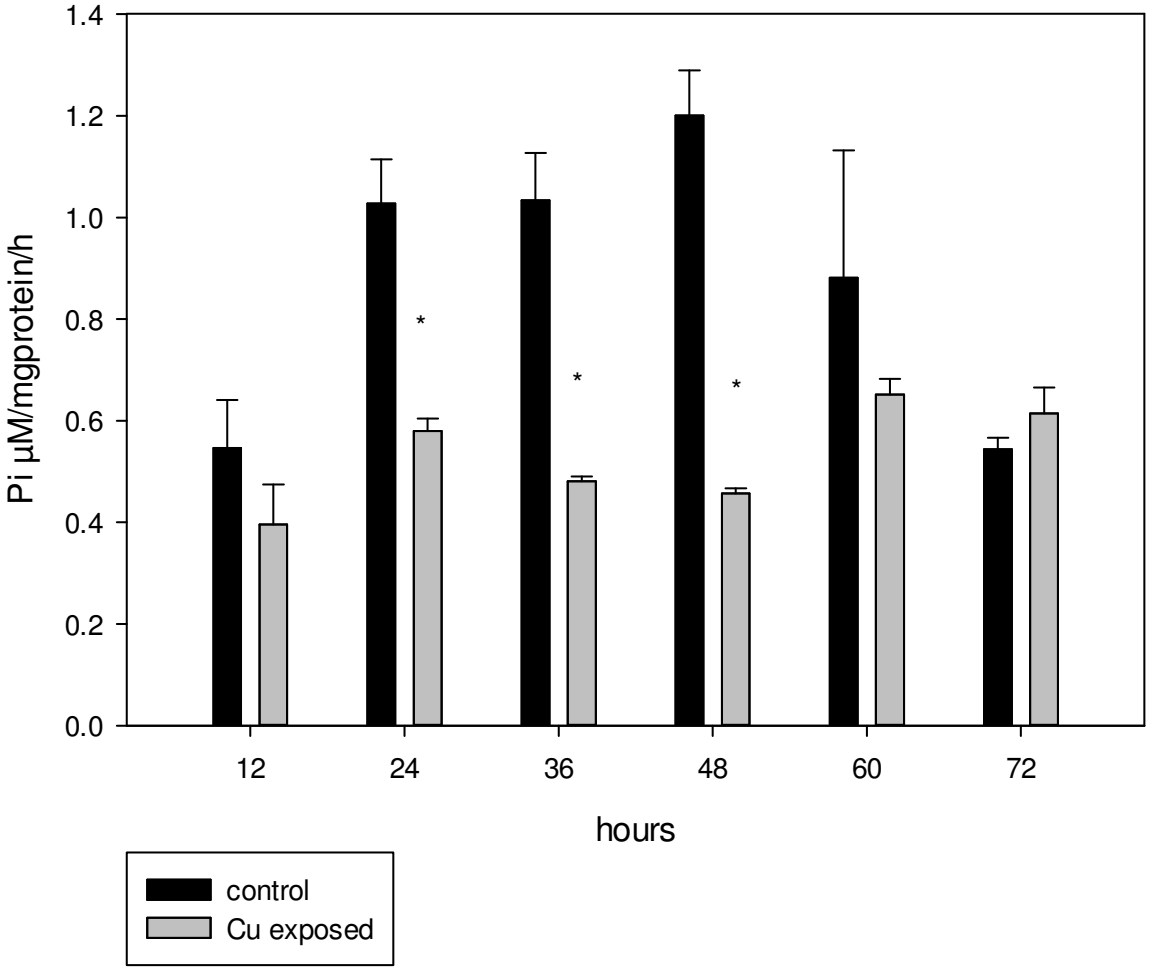

400
300
200
100
0
Mg mM/Kg
12
24
36
48
60
72
84
hours
control
Ni exposed

Figure 4-5 Ca ATPase activity measured every 12 hours over the first 72 hours of larval development in **a)** Cu exposed (17 µg/L) **b)** Ni exposed (33 µg/L). An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's t-test ($P<0.05$). Values are means ± SEM ($N = 4$).

a


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi µM/mgprotein/h
*
*
*
12
24
36
48
60
72
hours
control
Cu exposed

b

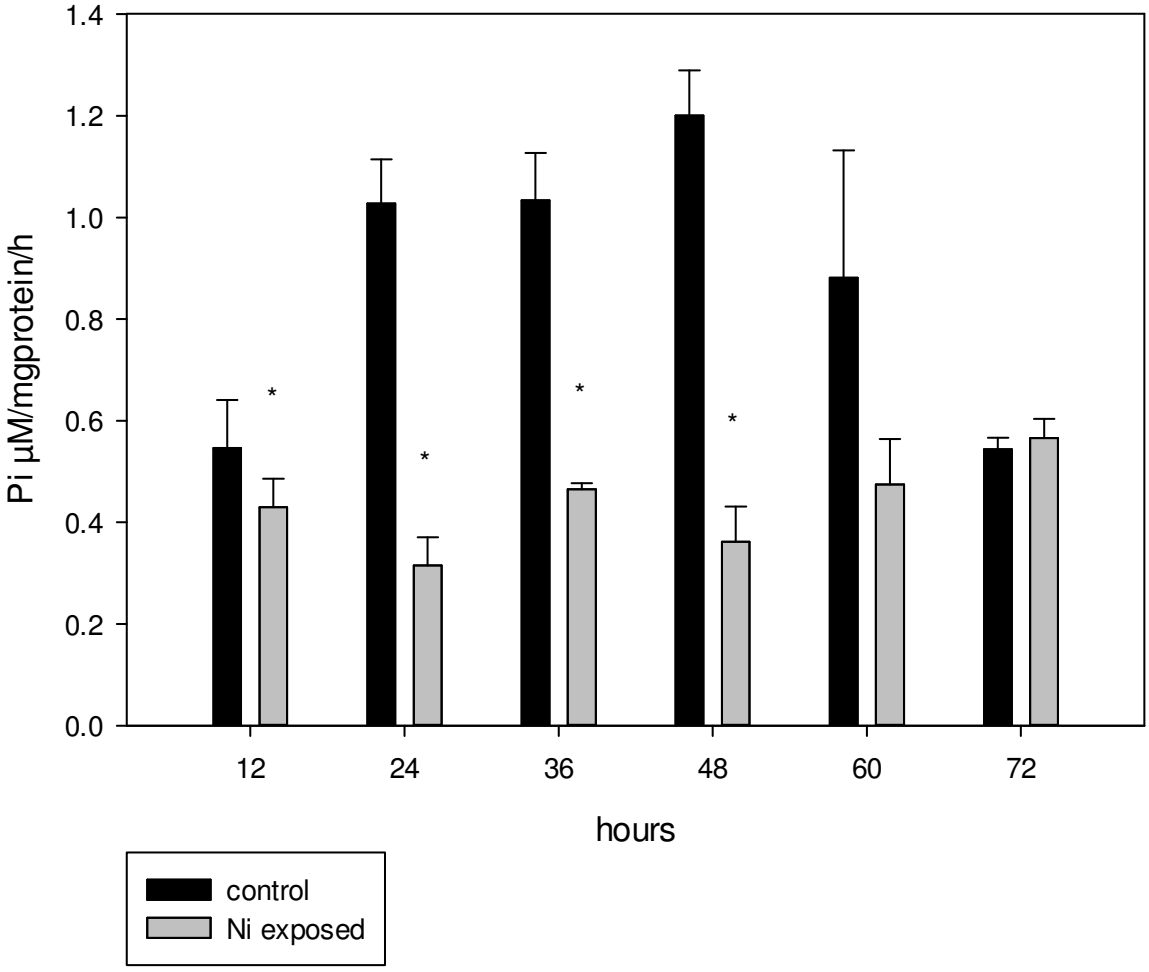
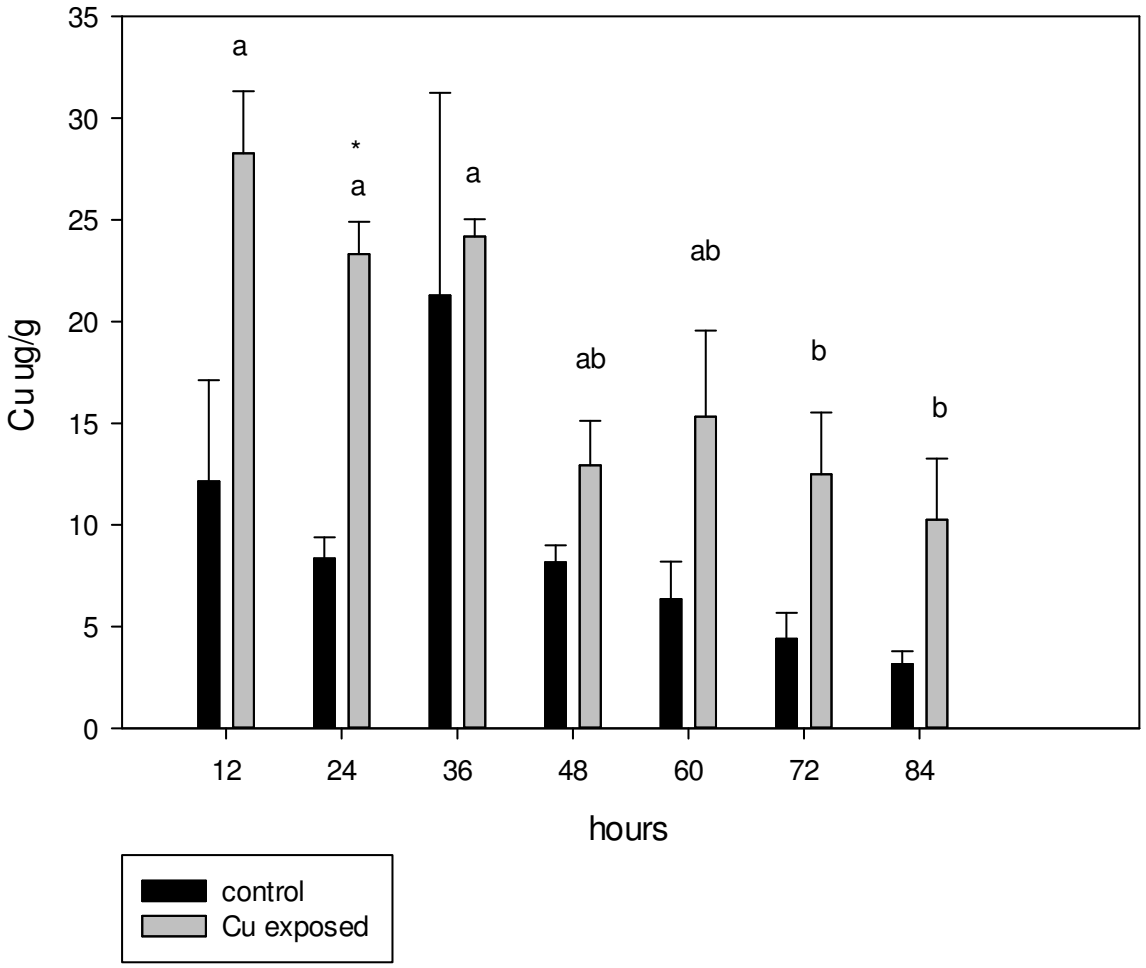

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pi µM/mgprotein/h
*
*
*
*
12
24
36
48
60
72
hours
control
Ni exposed

Figure 4-6. Whole body metal accumulation measured every 12 hours over the first 84 hours of larval development **a)** Cu exposed (6 µg/L) **b)** Ni exposed (47 µg/L). An asterisk (*) indicates a significant difference from control levels at the same time point as determined with a Student's t-test ($P<0.05$). Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Values are means ± SEM (N = 3-5).

a


35
30
25
20
15
10
5
0
Cu ug/g
a
*
a
a
ab
ab
b
b
12
24
36
48
60
72
84
hours
control
Cu exposed

b

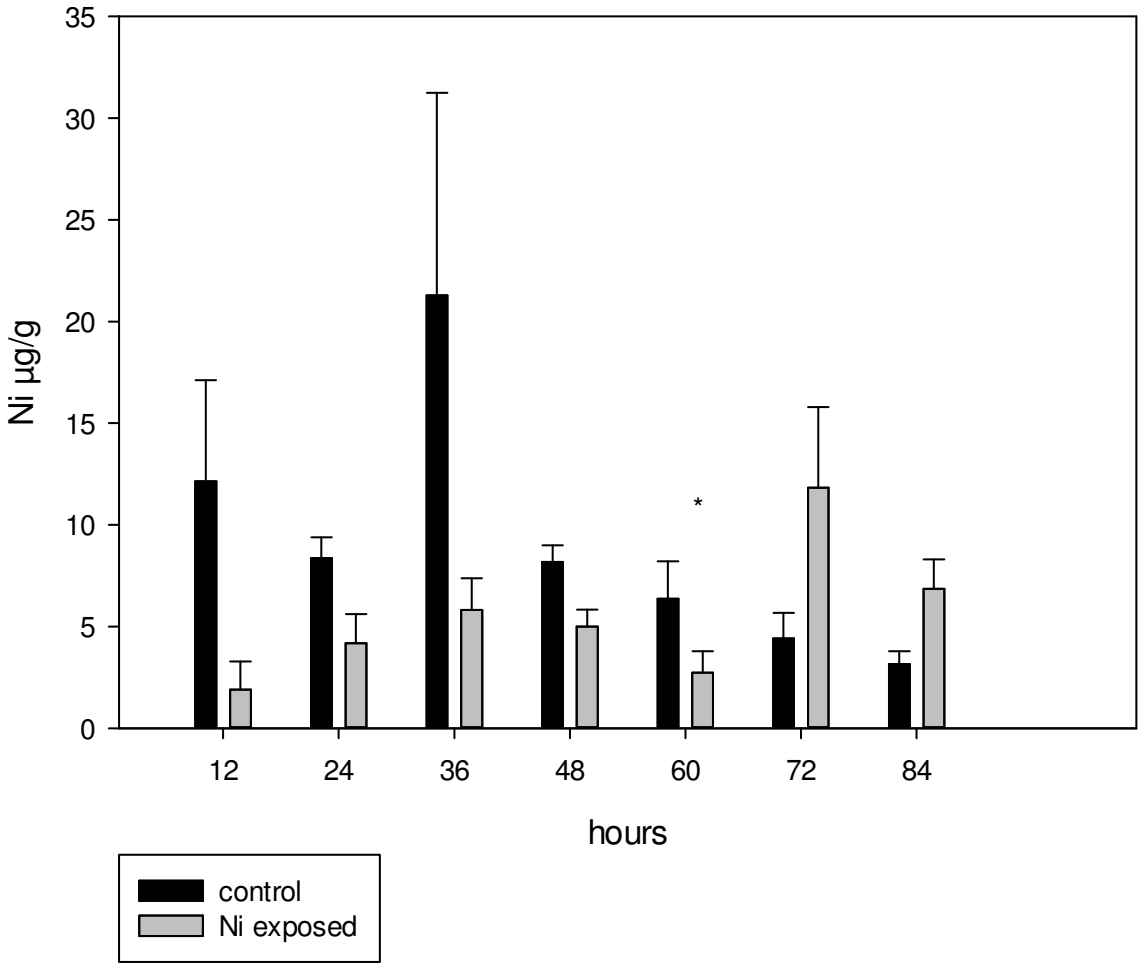
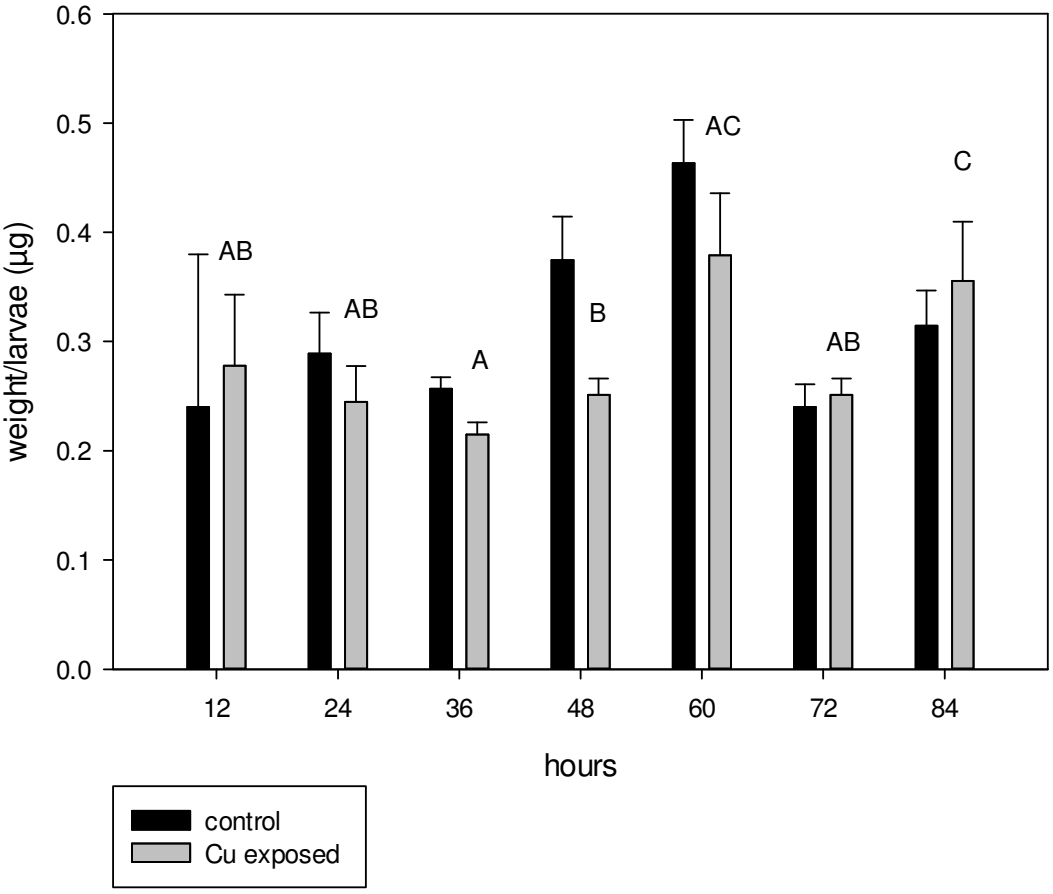
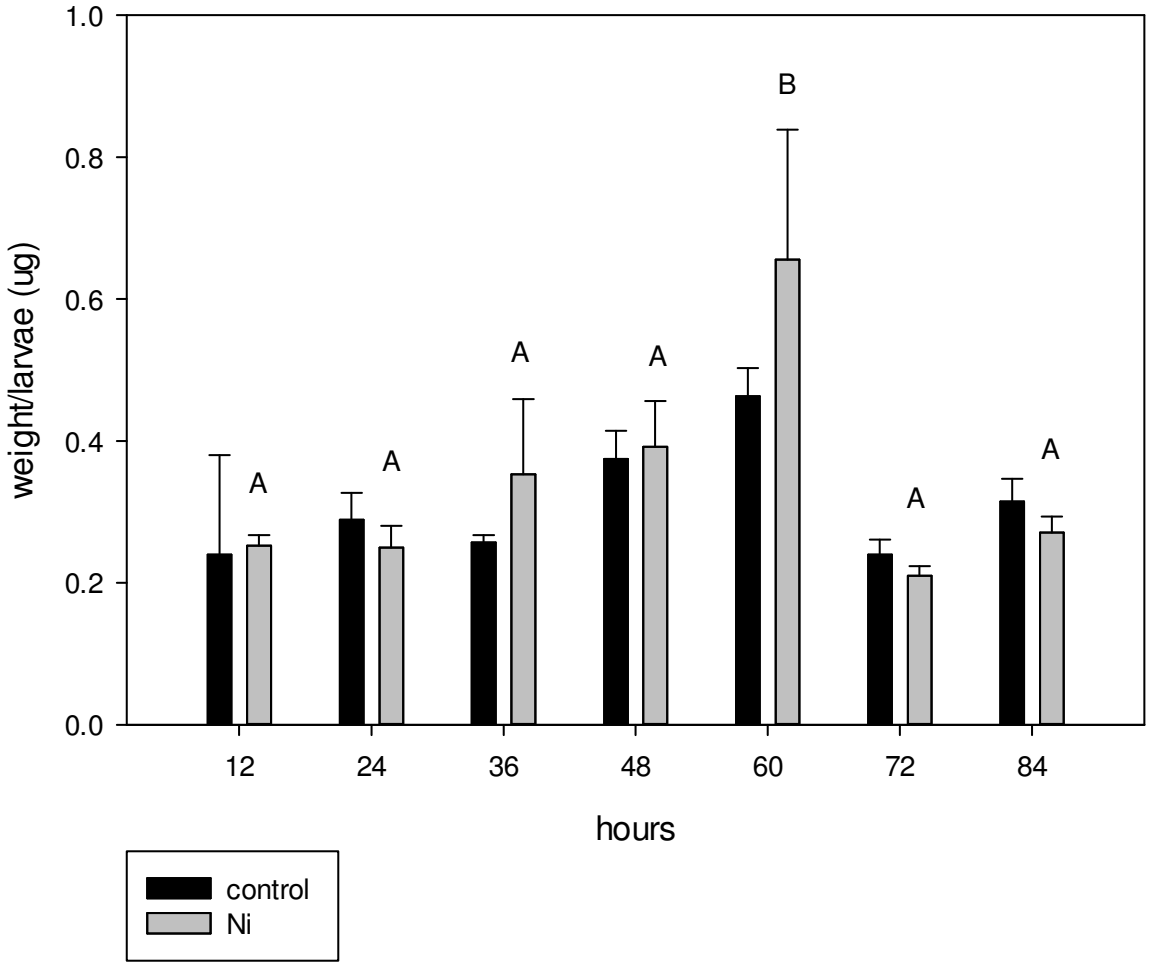

35
30
25
20
15
10
5
0
Ni µg/g
*
12
24
36
48
60
72
84
hours
control
Ni exposed

Figure 4-7. Larval weights measured every 12 hours over the first 84 hours of larval development in **a)** chronically Cu (6 ug/L) exposed larvae and **b)** chronically Ni (47 ug/L) exposed larvae. Values with different letters are significantly different as determined by an ANOVA followed by Fisher LSD post hoc. Values are means ± SEM (N= 3-5).

a


0.6
0.5
0.4
0.3
0.2
0.1
0.0
weight/larvae (µg)
AB
AB
A
B
AC
AB
C
12
24
36
48
60
72
84
hours
control
Cu exposed

b


1.0
0.8
0.6
0.4
0.2
0.0
weight/larvae (ug)
A
A
A
A
B
A
A
12
24
36
48
60
72
84
hours
control
Ni

CHAPTER 5: CONCLUSION

The objectives outlined in chapter 1 were fulfilled through various experiments. The specified objectives and resulting details follow:

To investigate the early developmental Ca profile of sea urchin embryos and larvae during the first 96 h development and develop a comparison of the different stages of the first 96 h of development. Here, the basic hypothesis was that stages which exhibit the most pronounced changes will be especially vulnerable targets of metal toxicity.

We conclude that Ca uptake, storage and utilization in the developing sea urchin embryo is highly variable in its temporal pattern over development and therefore can be capitalized upon as a potentially sensitive endpoint in toxicity testing. In particular the gastrulation stage of development showed the greatest increase in Ca uptake and accumulation, as it is a key stage in skeletogenesis. This led to our hypothesis that it could be a particular sensitive stage of development in which to study the effects of toxicants, particularly Ca disruptors such as metals. This hypothesis was confirmed in Chapters 3 and 4. Our findings will hopefully aid in developing sensitive endpoints in order to refine current toxicity tests utilized in environmental quality testing and the development of water quality guidelines.

To determine patterns in accumulation of other major cations (Mg, K and Na) in the early stages of embryonic and larval growth

An increase in Mg levels after gastrulation (48 h) during generation of the spicule was observed in our research (Figure 2-3d). Past work performed has shown the role of Mg in spicule formation. Mg is also structurally important in sea urchin development as $MgCO_3$ is found to be a significant component of the spicule, constituting 5% of its mineral phase (calcite, $CaCO_3$ is the predominant constituent) (Decker and William, 1988). The increase in Mg accumulation at this time confirms its role in spicule formation.

K and Na measurements were made all throughout embryonic development and the two ions displayed similar patterns in their accumulation (Figure 2-3b and c). These two ions have been implicated in amino acid transport. Therefore it would be of interest to perform further studies to investigate whether the variation in Na and K content also corresponds with amino acid transport and protein synthesis in the developing embryo and whether this might explain the accumulation pattern of these ions over development.

To determine median threshold values (EC50 and LA50) for Pb and Zn as well as the relative ionoregulatory disturbances associated with the varying amounts of metal accumulated in the larvae.

Sea urchin embryos were very sensitive to Zn with an EC50 of 2.3 µmol/L (95%

C.I. = 1.97–2.71) μmol/L and LA50 of 4.8 (2.16-11.33) μmol/g. Embryos displayed even higher sensitivity to Pb with an EC50 of 0.36 (0.25-0.49) μmol/kg and LA50 of 1.92 (1.67-2.78) μmol/kg. This is consistent with the principles of the BLM in that Pb with a higher log K value of 6 compared to a log K value of 5.3-5.5 for Zn (freshwater values reviewed by Niyogi and Wood, 2004) results in a lower EC50 and LA50 than Zn. Put simply Pb is more toxic than Zn, as less Pb is required to invoke equal toxicity. This toxicity is a reflection of the higher affinity (log K) of Pb for the biotic ligand.

To explore the effects of DOC on Zn toxicity

Initial research performed by Arnold (2005) on the interaction of Cu and DOC provided promising evidence of the protective properties of DOC against metal toxicity. The present research however, shows evidence to the contrary when Zn is combined with DOC. Brazilian inshore DOC was shown to increase the toxicity of Zn to sea urchin embryos, evident from a decrease in the Zn EC50 from 151 μg/L to 13.7 μg/L when 3 mg/L of the DOC was added. The EC50 was further decreased to 10.8 μg/L upon addition of a higher concentration (12 mg/L) of the same type of DOC; however this was not a large enough decrease to indicate dose dependent toxicity of the DOC in combination with Zn. Nordic reservoir DOC was more toxic than Brazilian inshore DOC as there was no survival of embryos at any concentration of Zn upon its addition to the Zn exposure. Interestingly, DOC in itself proved to be toxic to the embryos, as there was less survival in controls with Brazilian inshore DOC added, than controls without DOC. Nordic reservoir DOC again proved to be more toxic than Brazilian inshore DOC with no survival of embryos even in the absence of Zn (Figure 3-3a and b).

To determine the salinity threshold for developing sea urchin larvae

Sea urchin larvae were shown to be extremely sensitive to changes in salinity with less than 80% survival at 90% sea water (Figure 3-1). Echinoderm larvae in general are very poor ionoregulators and are viable only within a narrow margin of salinities (Kinne, 1971). Developing urchin embryos are able to maintain ion homeostasis by modifying cell membrane potential, which regulates cell permeability to ions. However ionoregulation only occurs within a narrow range of salinities as passive diffusion of ions into the embryo through transporters and voltage-gated channels is highly influenced by the concentrations of ions in the external medium (Hagiwara and Jaffe, 1979). As larvae were extremely sensitive to changes in salinity and DOC proved to be toxic, these environmental parameters were not altered for the remainder of the research.

To determine the effects of Pb, Zn, Cu and Ni on the Ca uptake profile during the first 96 h of development and investigate whether ionoregulatory disruption is a mechanism of toxicity of these metals through measuring ions over development in exposed larvae as well as over a range of concentrations of metals at the 72 h time point

Our research in chapters 3 and 4 shows that sea urchin embryos that an important mechanism of toxic action of chronic Pb, Zn, Cu and Ni exposure, is ionoregulatory disruption. In particular, a significant disturbance of Ca homeostasis was evident from an

inhibition of unidirectional Ca uptake rates, Ca accumulation in the whole organism, as well as Ca ATPase activity. This toxic effect is of notable significance as Ca is vital to sea urchin embryos in its roles in cell division and development of the spicule (skeleton). Indeed greater effects of metal stress on Ca homeostasis were observed at the skeletogenic gastrulation stage relative to other stages of early development. Acute metal challenge experiments however, demonstrated that this was not by a direct competition of Pb, Zn, Cu and Ni for the Ca uptake mechanism. While all four metals rendered their toxic action primarily through a disruption of Ca homeostasis, Cu and Ni had a greater overall effect on ionoregulation, evident from the disruption of K, Na and Mg levels measured.

Interestingly, the larvae display some capacity for recovery from toxicant stress as is evident from the return to normalcy of Ca uptake rates, Ca accumulation and Ca ATPase activity periodically over development. Toxic effects of metal exposure seen at earlier time points during development were sometimes not apparent at 72 h of development, the endpoint of standard toxicity tests used routinely in environmental monitoring. We propose measuring endpoints of toxicity periodically over early development as a more effective way of studying the toxic stress of contaminants. We also recognize that although certain toxic effects observed earlier were not apparent at 72 h, toxic effects may be still be present in biological endpoints that we did not analyze. Additionally compensatory mechanisms to combat metal uptake, such as a down regulation of DMT1 may affect the later health of urchins as uptake of essential metals may also be indirectly affected.

Effects of metal exposure that were observed in this study occurred at metal concentrations higher than the marine WQC for various countries described in Chapter 1. This indicates that countries with established marine WQC have set regulations at sufficient levels to ensure the protection of the sensitive organism, *S. purpuratus*. There is still much to be done in the way of metals toxicity research in the marine environment as many nations including Canada (with the exception of British Columbia) have yet to establish marine WQC. Ultimately, we hope that our data on the mechanisms of toxicity of Pb, Zn, Cu and Ni in seawater will contribute to the growing body of information on the toxicity of these metals in the marine environment. This will aid in the development of accurate marine water quality guidelines.

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